Functional characterisation of the hypertrehalosaemic hormone from the Indian stick insect *Carausius morosus*: metabolic and myotropic studies

by

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Plagiarism declaration

I, Ottilie Kandiwapa Hasiike Katali, know the meaning of plagiarism and declare that all of the work in this dissertation, save for that which is properly acknowledged, is my own.

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13 March 2017

Abstract

Neuropeptides of the adipokinetic hormone/red pigment concentrating hormone (AKH/RPCH) family are well known as regulators for many physiological processes in insects, notably energy metabolism, and a possible role in myostimulation. The Indian stick insect *Carausius morosus* contains two members of this family, hypertrehalosaemic hormone I and II (Carmo-HrTH-I and -II). Both these are decapeptides and they differ only at position 8, where the tryptophan of Carmo-HrTH-I is C-mannosylated.

It is known that Carmo-HrTHs increase the carbohydrate (trehalose) concentration in the haemolymph via a G protein-coupled receptor. The current study seeks to identify which part of the HrTH amino acid sequence is necessary to interact with the receptor on the fat body of *C. morosus* to trigger a response (hypertrehalosaemia) eventually leading to the release of carbohydrates into the haemolymph. In addition, the role of Carmo-HrTHs in stimulating the heart rate through myostimulation was also investigated.

Two biological assays were used to assess the potencies of various analogues, in comparison to that of the native peptides: (1) the carbohydrate-mobilizing assay assessed the increase in levels of carbohydrates; and (2) the semi-exposed heart assay assessed the increase in heart rates. The current study confirmed that both Carmo-HrTH-I and -II are capable of increasing the haemolymph carbohydrates in ligated stick insects. The results of the current study revealed, for the first time, that these peptides also similarly stimulate the heart rate of the stick insect. Thus, Carmo-HrTH-II was used as a lead peptide in the current study on which various naturally-occurring AKH peptides and systematically altered analogues were based.

The selected naturally-occurring AKH peptides had a single or double amino acids replacement in comparison to Carmo-HrTH-II and some were octapeptides. Each systematically altered analogue of the native *Carausius* HrTH II had a single amino acid replaced with alanine. Additionally, two analogues that lacked the N-terminal pyroglutamate residue or had a free threonine acid at the C-terminus instead of an amide were also tested.

The results showed that the N- or C- terminal modified analogues have no hypertrehalosaemic activity in *C. morosus* and are also incapable of increasing the heart rate of this insect as high as Carmo-HrTH-II. This suggests that the blocked termini are important features, for both peptide protection and receptor binding. The structural requirements of *C. morosus* receptor(s) for Carmo-HrTHs appear to be very specific. The receptor(s) do not accept octapeptides and only four out of the fourteen decapeptides elicited at least 46% of the biological activity as compared to the native peptide and the rest (ten) were not active. This implies that the HrTHs receptor (s) for *C. morosus* do not tolerate the replacement of most single amino acids. In the heart assay, known cardio-stimulatory peptides were applied to the semi-exposed heart of *C. morosus* to establish the potential extent of cardioexcitation. The results of this study revealed that crustacean cardioactive peptide and proctolin are capable of increasing the heart beat rate of the stick insect more than the AKH/RPCH peptides. It was also shown that the stick insect heart beat rate can be inhibited by octopamine.

This is the first study to investigate, in detail, the importance of structural features of the hypertrehalosaemic hormones of the Phasmatodea insects, and is therefore an important contribution to designing environmentally friendly insect-specific pesticides.

Dedication

I did not get a chance to know my father, he passed when I was about 3 years old. Sadly, he left me with no siblings. Seven years later, my mother passed away, leaving me with one younger sister, Emilia Ndilimeke Endjambi. Ever since the death of our mother, I kept telling myself that I am now the new mom to my sister and I have to work hard to be able to support us both. Whenever I feel like giving up, the thoughts of my sister always push me out of it. For my sister, I managed to push through many challenges in life including making it to the end of this research project. In this spirit, I am entirely dedicating this dissertation to Ndilimeke for being a very important piece of life. "*Ndilimeke, ondikuhole nkelo hameme niiningwanima yomuuyuni mbuka otuna okuyi taalela atushe*."

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1. Chapter one: Introduction

1.1. Background

Insects are a major class of invertebrates within the phylum Arthropoda and make up the majority of animal species on earth (Foottit & Adler, 2009; Capinera, 2010). In addition to their vital role in ecosystem functioning, they have many economic benefits for society, such as food resources, especially in Africa, Asia, Australia and Latin America (e.g., locusts and moth larvae), medical treatments (e.g., maggot therapy using fly larvae) and dyes and silk production (e.g., scale insects and silk moths) (Capinera, 2010). Their ecological functions are varied, from pollination (e.g., bees and butterflies) to feeding on organic waste or dead organisms (e.g., beetles and blow fly larvae); most importantly, they are critical components of food webs (Capinera, 2010; Gullan & Cranston, 2014).

However, insects are not always beneficial and impact humans in various ways. They can be pests of stored products (e.g., cockroaches) or crops (e.g., grasshoppers), parasites on domesticated animals (e.g., lice), and they also have significant medical impacts, contributing towards the spread of diseases, such as malaria and yellow fever (Burn *et al.*, 1987; Capinera, 2010; Gullan & Cranston, 2014).

Insects that are harmful to humans need to be regulated and their populations controlled. Currently, there are three methods of regulating insect numbers: (1) physical procedures – using thermal and mechanical shock, electromagnetic radiation, pneumatic and other types of physical barriers, (2) biological methods – using other organisms to eliminate or reduce the population of targeted insects, (e.g., introducing an enemy, pathogen or parasitoid into the environment to act against the pest insect) and (3) chemical methods – using chemicals to eradicate target insects (reviewed by Wojciechowska *et al.*, 2016).

Most synthetic chemical insecticides either act as enzyme inhibitors (e.g., organochlorines and organophosphorus), which disrupt various metabolic processes, such as oxidative phosphorylation, or inhibit specific neurophysiological processes, such as axonal transmission (Burn *et al.*, 1987). Insecticides are mainly used by farmers for crop protection (Tisdell, 2001; Schreinemachers & Tipraqsa, 2012). Unfortunately, uncontrolled applications of chemical insecticides have resulted in the evolution of insect resistance as well as in the contamination of the environment. Toxic residues of insecticides have major ecological implications and are a threat to human health (see reviews Altstein *et al.*, 2000; Mansouri *et al.*, 2017).

Agriculture is now turning towards environmentally friendly insecticides that are selective and affect only the target species instead of all insects in the ecosystem, which would include valuable insects that pollinate and provide a food source for insectivorous animals or humans (Altstein *et al.*, 2000; Gäde & Goldsworthy, 2003). One idea of tackling this is through the use of neuropeptides, which regulate most of the key physiological processes like embryonic development, homeostasis and osmoregulation in insects. Hence, developing non-toxic and species-specific insecticides by designing specific peptidomimetics to act as antagonists of insect neuropeptides would minimize the environmental impact (Altstein *et al.*, 2000).

The first step towards developing specific, neuropeptide-based antagonists is to have a knowledge of the range of neuropeptides and their regulatory functions in target species. Therefore, this study focused on some of the neuropeptides involved in critical physiological processes (i.e., metabolism and circulatory system) in stick insects. Knowing the peptide sequence in a particular species and its performance in different biological assays allows for

predictions of the conformation of the receptors and binding sites. Hence, the knowledge generated by such a study, together with similar studies performed previously on other insects, such as cockroaches, flies, locusts and moths (Baumann *et al*., 1990; Ziegler *et al.*, 1991, 1998; Poulos *et al.*, 1994; Gäde & Hayes, 1995; Goldsworthy *et al.*, 1997; Caers *et al.*, 2012, 2016; Marco & Gäde, 2015), will assist in the development of hormone-like compounds that can be used in specific drug design to act as pesticides.

1.2. The insect endocrine system

In insects, the endocrine system plays a major role in regulating various physiological processes, such as metabolism, homeostasis, circulation, growth and reproduction. The insect endocrine system is mainly made up of (1) the brain and associated corpora cardiaca/corpora allata (CC/CA) complex, which consist of neurosecretory cells and (2) the prothoracic gland together with several other groups of endocrine cells found in the neural ganglia, gut, gonads and epidermis (reviewed by Gäde *et al.*, 1997; Nijhout, 1998).

The three main chemical classes of hormones synthesised and secreted by the insect endocrine system are juvenile hormones, ecdysteroids and neuropeptides (Gäde *et al.*, 1997; Nijhout, 1998). The first class, juvenile hormones, are acyclic sesquiterpenoids hormones produced and secreted by the corpora allata (CA), a small pair of organs connected to the corpora cardiaca (CC) (Nijhout, 1998). These hormones regulate metamorphosis and control reproduction; however, they are also involved in other processes, such as insect behaviour, diapause and caste determination in certain insects (Nijhout, 1998). The second class, ecdysteroids, are steroid hormones mainly produced by the prothoracic glands and partly by the gonads. These hormones regulate principal events in the insect life, such as development, metamorphosis, moulting and reproduction (Nijhout, 1998).

The third class, neuropeptides (peptide hormones), are produced by neurosecretory cells found in the brain, CC and throughout the nervous system (Gäde *et al.*, 1997; Nijhout, 1998). The CC are the principal neurohemal organs that store the products of the neurosecretory cells of the brain. The CC also contains intrinsic neurosecretory cells that synthesise and release their peptide hormones locally (Gäde *et al.*, 1997). Neuropeptides are the main regulators of behavioural, physiological and biochemical processes in the insect body (Gäde *et al.*, 1997).

1.3. Structure and function of neuropeptides

Neuropeptides are short chains of α-amino acids linked by an amide bond that are encoded within the genome in the form of larger precursor proteins known as prepro-hormones. In most cases, multiple peptides are contained within a precursor protein and each peptide is surrounded by cleavage sites. However, there are few cases where the precursor protein contains only one peptide (reviewed by Gäde *et al*., 1997; Nässel, 2002).

Reviews by Nässel (2002) and Zhang et al., (2010) describe that after translation, the prepro-hormone enters the secretory pathway. This pathway involves further processing, maturation and occasional sorting. The peptides undergo extensive post-translational processing, which results in modifications such as carboxy (C)-terminus amidation, cyclisation of N-terminus glutamine/glutamic acid residues, sulfation of tyrosyl groups and disulfide bridging between cysteines. Frequently, these post-translational modifications are responsible for a peptide assuming its bioactive conformation (Nässel, 2002). Thereafter, the peptides are packed into vesicles and transported to the release site for storage until the time of discharge to targets. The targets can be (1) the synthesising neuron itself, (2) tissues that are near the synthesising neuron, or (3) tissues that are far from the point of release, in which case the peptide is transported via the circulatory system (Nässel, 2002).

1.3.1. Adipokinetic hormone/red-pigment concentrating hormone (AKH/RPCH) peptide family

1.3.1.1. The origin of the AKH/RPCH family

One of the neuropeptides that has this structure, function and distribution is the AKH/RPCH family. This family of neuropeptides often occurs in invertebrates. It is best-studied in Insecta and Crustacea (see review by Gäde, 2009) but also in other invertebrate phyla including Annelida, Mollusca and Nematoda (Lindemans *et al.*, 2009; Hauser & Grimmelikhuijzen, 2014; Johnson *et al.*, 2014; Li *et al.*, 2016). In decapod crustaceans and insects, these neuropeptides are produced by the X-organ cells in the eyestalks and the intrinsic neurosecretory cells of the CC, respectively (see reviews Nijhout, 1998; Gäde & Marco, 2013). The name of the family (adipokinetic hormone/red-pigment concentrating hormone family) is derived from the functions of the first two members.

RPCH was the first member of the AKH/RPCH family to be discovered. Extracts from certain glands of crustaceans and insects were found to have effects on changing shrimp colouration from dark to light. This was determined to be a result of aggregation of pigment granules in the epithelial chromatophores and distal pigment cells in the eye (reviewed by Rao, 2001; Gäde & Marco, 2009). In 1972, one of the neuropeptides responsible for this colour change in crustaceans was identified. It was isolated from the eyestalks of the prawn *Pandalus borealis* (Fernlund & Josefsson, 1972) and was given the acronym Panbo-RPCH. This first member of the AKH/RPCH family was also the first neuropeptide to be fully characterised in invertebrates. The sequence (Fig. 1.1) was elucidated by a combination of mass spectrometry and Edman-dansly analysis (Fernlund & Josefsson, 1972).

In 2003, the existence of Panbo-RPCH in insects was reported for the first time (Gäde *et al.*, 2003). This peptide was isolated from the CC of the hemipteran insect *Nezara viridula*, and it

is responsible for the release of stored lipids from the fat body, a process known as the adipokinetic effects (Gäde *et al.*, 2003). To date, Panbo-RPCH is known to occur in several malacostracan crustaceans and in various insect orders, including Coleoptera, Hemiptera and Plecoptera (reviewed by Gäde & Marco, 2015).

Other forms of RPCH that differ from Panbo-RPCH at three positions (Fig. 1.1) are reported in more primitive crustaceans: (1) Dappu-RPCH, found in *Daphnia* species (Christie *et al.*, 2008; Marco & Gäde, 2010) and (2) Argsi-RPCH, predicted to occur in the parasitic crustacean *Argulus siamensis* (Christie, 2014).

Panbo-RPCH	pGlu	Leu	Asn	Phe	Ser	Pro	Gly	Trp	amide
Dappu-RPCH	pGlu	Val	Asn	Phe	Ser	Thr	Ser	Trp	amide
Argsi-RPCH	pGlu	Val	Asn	Phe	Ser	Thr	Lys	Trp	amide

Figure 1.1. Primary sequences of Panbo-RPCH, Dappu-RPCH and Argsi-RPCH (Fernlund & Josefsson, 1972; Marco & Gäde, 2010; Christie, 2014). Residues highlighted differ from those in Panbo-RPCH.

In the 1960's, a second member of the AKH/RPCH family was discovered. The CC of the American cockroach, *Periplaneta americana*, and the locusts, *Locusta migratoria* and *Schistocerca gregaria*, were found to have the ability to elevate the concentrations of carbohydrates and lipids in the haemolymph of the cockroach and the locusts, respectively (Steele, 1961; Beenakkers, 1969; Mayer & Candy, 1969). In 1976, one of the peptides responsible for mobilising the haemolymph lipids (i.e., adipokinetic effects) in *L. migratoria* and *S. gregaria* was isolated and sequenced from the CC of these species (Stone *et al.*, 1976). The sequencing was achieved in a method similar to the one used to sequence

Panbo-RPCH. The resulting insect neuropeptide is currently known as Locmi-AKH-I (Table 1.1), and it is closely related to Panbo-RPCH.

Later, two more AKHs were isolated from the CC of *L. migratoria* (Siegert *et al.*, 1985; Gäde *et al.*, 1986; Oudejans *et al.*, 1991) and one more from *S. gregaria* (Siegert *et al.*, 1985; Gäde *et al.*, 1986). Furthermore, two AKHs were isolated from the CC of *P. americana* (Baumann & Penzlin, 1984; Scarborough *et al.*, 1984; Witten *et al.*, 1984; Siegert & Mordue, 1986). All of these peptides were found to be structurally related to Panbo-RPCH and Locmi-AKH-I. There have been many other discoveries of AKHs from various insect orders, with many individuals containing two or three of these peptides (Gäde, 2009). The maximum number of AKHs found in any individual is five, reported in striped hawk moths, *Hippotion eson* and *H. celerio* (Gäde *et al.*, 2013).

The isolation of the AKHs from most insects involves extracting peptides from the CC using 80% methanol. These peptides are then purified from the methanolic CC extract using a single-step reversed phase high-performance liquid chromatography (RP-RPCH) and targeted using biological assays. However, if whole heads are used as starting material instead of CC, then, many more purification procedures are required due to impurities. The primary sequences of the purified AKHs, i.e., the active RP-HPLC fractions, are then identified by a combination of Edman degradation techniques and mass spectroscopy (Gäde *et al.*, 1997). In recent years, the primary sequences of AKHs have also been achieved by directly analysing the CC peptide extract with a liquid chromatography-electrospray ionisation ion trap mass spectroscopy (Kodrík *et al.*, 2010; Gäde & Marco, 2011, 2012).

1.3.1.2. The characteristics of AKHs

An AKH prepro-hormone typically consists of a signal sequence, an immature AKH peptide, several processing sites and an AKH-precursor-related peptide of unknown function (Gäde, 2004b). In certain cases where insects contain more than one AKH, such as *L. migratoria*, the AKHs are from prepro-hormones translated from separate mRNAs (Gäde, 1997a; Van der Horst, 2003).

The primary sequences of AKH/RPCH peptides generally consist of 8-10 amino acids with blocked termini, i.e., having pGlu at the N-terminus and a carboxy amide at the C-terminus (reviewed by Gäde, 2009), which protect the peptide against exopeptidases. Most of these peptides have two aromatic amino acids, one at position 4 (mostly Phe and a few Tyr) and the other at position 8 (Trp). However, there are a number of cases where the AKHs have more than two aromatic acids. For example, AKHs isolated from scarab beetles have three aromatic amino acids at position 2 (Phe), 4 (Tyr) and 8 (Trp) (Gäde, 2009). Additionally, these peptides have a hydroxylated residue at position 5 (Ser or Thr) and longer peptides have a Gly at position 9 (Gäde, 2009). The majority of AKH/RPCH peptides are neutral (Gäde, 2009), with the exception of those that have a charged Asp or Lys residue at position 6 or 7 (Marco *et al.*, 2013; Christie, 2014). See Table 1.1 for the primary sequences of several members the AKH/RPCH family.

In addition to the post-translational modifications of the termini, there are extra post-translational modifications reported in some insects. The Carmo-HrTH-I of the stick insect*, C. morosus*, has a C-mannosylated Trp at position 8 (Gäde *et al.*, 1992; Munte *et al.*, 2008). *Baculum extradentatum* has a peptide of the same design as Carmo-HrTH-II, except its Trp^8 is modified to a kynurenine residue instead (Malik *et al.*, 2012), the function of which has not yet been determined. In the Protea beetle, *Trichostetha fascicularis,* the Thr^6 residue of

Trifa-CC is phosphorylated (Gäde *et al.*, 2006). Also, the Nezvi-AKH of the stink bug *Nezara viridula* has a hydroxyproline at position 6 (Gäde *et al.*, 2011).

The conformations of AKHs have been investigated in a number of studies using nuclear magnetic resonance and molecular modelling techniques (Zubrzycki & Gäde, 1994, 1999; Nair *et al.*, 2001; Jackson *et al.*, 2014). These studies reported that the N-terminal residues of AKHs have adopted beta sheet structures, and there is an existence of a beta-turn around the amino acids at position 4 to 8. Additionally, Mugumbate *et al.* (2013) discovered that the AKH from the mosquito, *Anopheles gambiae,* has an almost cyclic conformation that is supported by a hydrogen bond between the Thr^3 and C-terminal. These authors further report that, upon binding to its receptor, this hydrogen bond is broken, causing the molecule to adopt to an extended structure.

Table 1.1. A selection of peptides from the AKH/RPCH family. Source: Gäde, 2009.

Peptide name	Primary sequence											Source
Aedae-AKH	pGlu	Leu	Thr	Phe	Thr	Pro	Ser	Trp	amide			Diptera: *Aedes aegypti* and *Culex pipiens;* Megaloptera: *Sialis lutaria.*
Locmi-AKH-II	pGlu	Leu	Asn	Phe	Ser	Ala	Gly	Trp	amide			Orthoptera: *Locusta migratoria and Locustana pardalina*
Locmi-AKH-III	pGlu	Leu	Asn	Phe	Thr	Pro	Trp	Trp	amide			Orthoptera: *L. migratoria and L. pardalina*
Peram-CAH-I	pGlu	Val	Asn	Phe	Ser	Pro	Asn	Trp	amide			Blattodea: *Periplaneta americana*, *P. brunnea*, *P. australasiae* and 11 other species
Peram-CAH-II	pGlu	Leu	Thr	Phe	Thr	Pro	Asn	Trp	amide			Blattodea: *P. americana*, *P. brunnea*, *P. australasiae* and 11 other species
Pyrap-AKH	pGlu	Leu	Asn	Phe	Thr	Pro	Asn	Trp	amide			Hemiptera: *Pyrrhocoris apterus*, *Dysdercus intermedius*, *D. cingulatus* and 2 other species
Scade-CC-I	pGlu	Phe	Asn	Try	Ser	Pro	Asp	Trp	amide			Coleoptera: *Scarabaeus deludens*, *Garreta nitens*, *Onitis aygulus and O. pecuarius*
Scade-CC-II	pGlu	Phe	Asn	Try	Ser	Pro	Val	Trp	amide			Coleoptera: *S. deludens and G. nitens*
Manse-AKH	pGlu	Leu	Thr	Phe	Thr	Ser	Ser	Trp	Gly	amide		Lepidoptera: *Manduca sexta, Heliothis zea, Bombyx mori* and 8 other species Hymenoptera: *Apis mellifera ligustica*
Carmo-HrTH-I	pGlu	Leu	Thr	Phe	Thr	Pro	Asn	Trp*	Gly	Thr	amide	Phasmatodea: *Carausius morosus*
Carmo-HrTH-II	pGlu	Leu	Thr	Phe	Thr	Pro	Asn	Trp	Gly	Thr	amide	Phasmatodea*: C. morosus, Extatosoma tiaratum and Sipyloidea sipylus*
Locmi-AKH-I	pGlu	Leu	Asn	Phe	Thr	Pro	Asn	Trp	Gly	Thr	amide	Orthoptera: *L. migratoria*, *S. gregaria, Acrida acuminata* and 10 other species.
Phyle-CC	pGlu	Leu	Thr	Phe	Thr	Pro	Asn	Trp	Gly	Ser	amide	Orthoptera: *Phymateus leprosus and Bullacris discolor*
Phymo-AKH	pGlu	Leu	Asn	Phe	Thr	Pro	Asn	Trp	Gly	Ser	amide	Orthoptera: *Phymateus morbillosus* and *Dictyophorus spumans*
Rommi-CC	pGlu	Val	Asn	Phe	Thr	Pro	Asn	Trp	Gly	Thr	amide	Orthoptera: *Romalea microptera and Taeniopoda eques*

The asterisk (*) indicates that Trp is C-mannosylated and the grey highlights represent conserved residues.

1.3.1.3. The functions of AKHs

In insects, the AKHs are well known for their involvement in the regulation of energy metabolism. These peptides are also being reported as being responsible for other functions including the stimulation of the heart rate (Baumann & Gersch, 1982; Malik *et al.*, 2012), the stimulation of muscle contraction in an isolated leg of the locust (O'Shea *et al.*, 1984), the inhibition of protein, fatty acids and RNA syntheses in locusts (Carlisle & Loughton, 1986; Gokuldas *et al.*, 1988; Kodrík & Goldsworthy, 1995) and the regulation of digestion (Kodrík *et al.*, 2012; Stoffolano *et al.*, 2014).

1.4. Neuropeptide regulation of metabolism

Physiological processes such as metabolism are tightly regulated. Metabolism is the set of chemical processes taking place in a living cell and is the foundation for regulating the physiology of an organism. Metabolic reactions are responsible for the conversion of fuel to energy and the synthesis of larger molecules from smaller components (Brooker *et al.*, 2014).

In insects, neuropeptides from the AKH/RPCH family are the main regulators for metabolism. Specifically, they are responsible for the release of stored fuel metabolites into the haemolymph during times of extreme energy demand such as flight (Gäde, 2004a). Such metabolites can either be diacylglycerol, proline or trehalose, which are stored in the fat body in forms of triacylglycerol or glycogen (Gäde & Marco, 2013). In certain insects, such as locusts (Stone *et al.*, 1976) and moths (Gäde *et al.*, 2013; Weaver *et al.*, 2012), the AKHs increase haemolymph diacylglycerol while in others, such as cockroaches (Hayes *et al.*, 1986; Siegert & Mordue, 1986) and stick insects (Gäde & Lohr, 1982; Malik *et al.*, 2012), trehalose is increased. In beetles, AKHs cause proline to increase (Auerswald & Gäde, 1995; Gäde, 1997b). AKHs are usually referred to the specific increase of the fuel metabolites they

cause in the haemolymph of the first insect they were isolated from. Thus, (1) adipokinetic hormones (AKH) –increase lipid (diacylglycerol mobilisation); (2) hypertrehalosaemic hormones (HrTH) – elevates trehalose (Carbohydrate-mobilisation) and (3) hyperprolinaemic hormones – increase proline (proline-mobilisation) (Gäde & Marco, 2013). However, these peptides are usually referred to by their generic name, the adipokinetic hormones (AKHs).

AKHs are released by the insect from the CC into the haemolymph only when required, such as when insects are short of food or during the time of motor activity (Gäde 1997a). Some insects, such as *L. migratoria*, are known to have only a small fraction of the stored AKHs released into the haemolymph (Cheeseman & Goldsworthy, 1979), and all three types of AKHs of this locust are released (Oudejans *et al.*, 1996).

The common understanding is that AKHs are transported into the haemolymph without carrier proteins (Oudejans *et al.*, 1996). In *L. migratoria,* the half-lives of the AKHs, Locmi-AKH-I, Locmi-AKH-II and Locmi-AKH-III) are 51, 40 and 5 minutes respectively during resting and 35, 37 and 3 minutes respectively during flight (Oudejans *et al.*, 1996). Once these peptides are released from the CC, they are either transported to their target cells to bind to their respective receptors or broken down by peptidase and eliminated from the haemolymph (Gäde, 1997a).

The adipokinetic hormone receptors (AKHRs) belong to the G-protein-coupled receptor family and are structurally and evolutionally related to vertebrates' gonadotropin-releasing hormone receptors (Park *et al.*, 2002; Belmont *et al.*, 2006; Alves-Bezerra *et al.*, 2016). The first AKHRs were cloned from the fruit fly *Drosophila melanogaster* and the silkworm *Bombyx mori* (Park *et al.*, 2002; Staubli *et al.*, 2002). Subsequently, AKHRs were cloned or

predicted from the genome data of several insects, such as *P. americana* (Hansen *et al.*, 2006; Wicher *et al.*, 2006), *M. sexta* (Ziegler *et al.*, 2011), *Anopheles gambiae* (Belmont *et al.*, 2006; Kaufmann & Brown, 2006) and *Rhodnius prolixus* (Alves-Bezerra *et al.*, 2016).

Once the AKH binds to its specific receptor on the fat body cell, the binding results in the change in conformation of the receptor, which interacts with a guanine nucleotide-binding protein (G-protein). This results in the transduction of an extracellular signal into an intracellular signal to elicit biological effects (Gäde *et al.*, 1997).

The intracellular signalling of AKHs to increase the concentration of fuel metabolites in the haemolymph is reviewed by Gäde & Auerswald (2003). For insects that oxidise carbohydrates (trehalose), the AKH binds to its specific receptor, which activates the Gq- and Gs-proteins (Fig. 1.2). The activation of the Gq-protein results in the stimulation of phospholipase C, causing the second messengers, inositol triphosphate (IP_3) and diacylglycerol, to be synthesised. In turn, this stimulates the elevation of intracellular Ca^{2+} and the activation of protein kinase C, leading to the activation of glycogen phosphorylase. Finally, glycogen phosphorylase breaks down the glycogen to glucose-1-phosphate, which is then converted to trehalose and released into the haemolymph. When the Gs-protein is activated, it in turn activates adenyl cyclase which results in the production of the second messenger, cyclic AMP. This results in the activation of phosphorylase B kinase that converts glycogen to glucose-1-phosphate, which is then converted to trehalose (Gäde & Auerswald, 2003).

For the insects that oxidise lipids (diacylglycerols), the Gs-protein activates the stimulation of intracellular Ca^{2+} and cyclic AMP (Fig. 1.3). This, in turn, results in the activation of protein kinase A, which activates triacylglycerol lipase (TAG lipase). TAG lipase breaks down the

stored triacylglycerol to diacylglycerols and fatty acids. The diacylglycerols are then released into the haemolymph. For the insects that oxidise proline, fatty acids are further converted to acetyl-CoA and then to proline (Gäde & Auerswald, 2003).

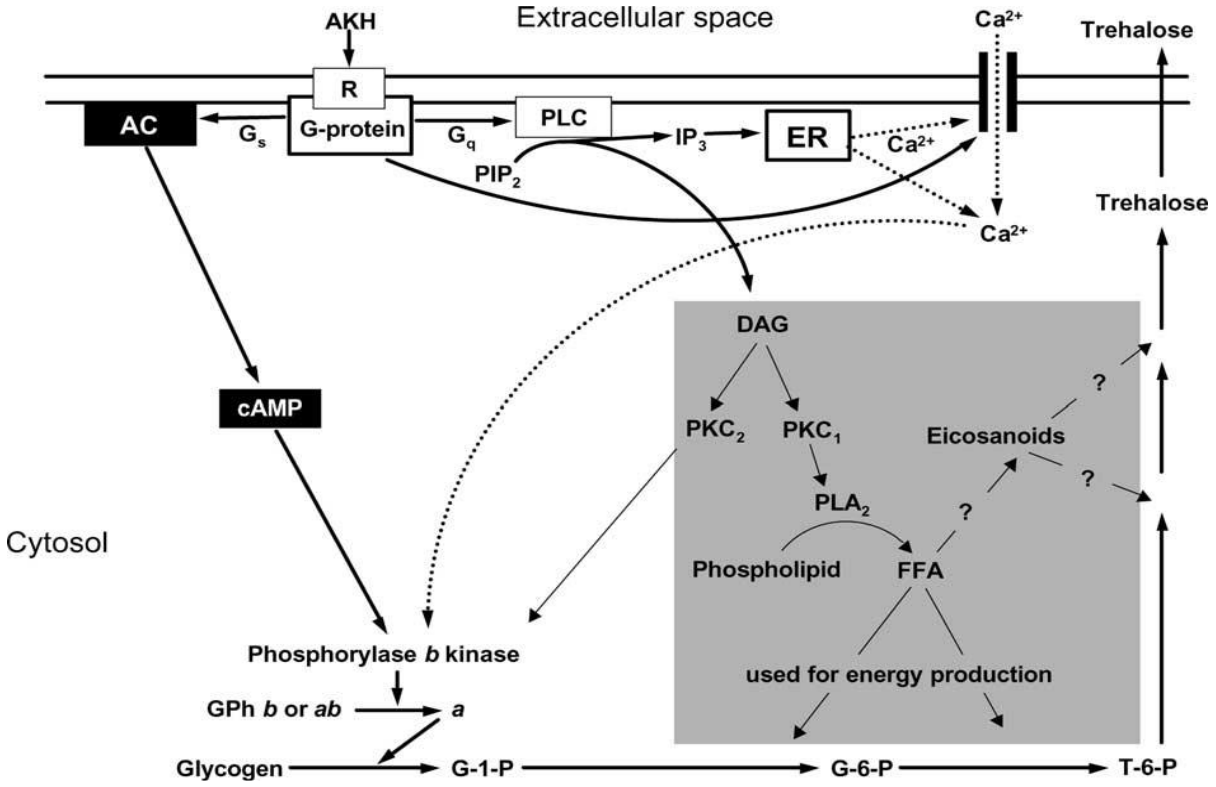

Figure 1.2. Proposed signal transduction pathway of the mobilisation of trehalose by AKHs in the fat body of *L. migratoria*, *P. americana*, *B. discoidalis* and *Pachnoda sinuata*. Abbreviations: adenylyl cyclase (AC), cyclic AMP (cAMP), diacylglycerol (DAG), endoplasmic reticulum (ER), free fatty acids (FFA), glucose-1-phosphate (G-1-P), glucose-6-phosphate (G-6-P), glycogen phosphorylase (GPh), inositol trisphosphate (IP_3), phosphatidylinositol bisphosphate (PIP_2) protein kinase A (PKA) PKC, protein kinase C (PKC), phospholipase C (PLC), receptor (R) and trehalose-6-phosphate (T-6-P). White letters on a black background indicate the pathway in locusts only. Events in grey area are only researched in and proposed for *P. americana*. Source: Gäde & Auerswald (2003).

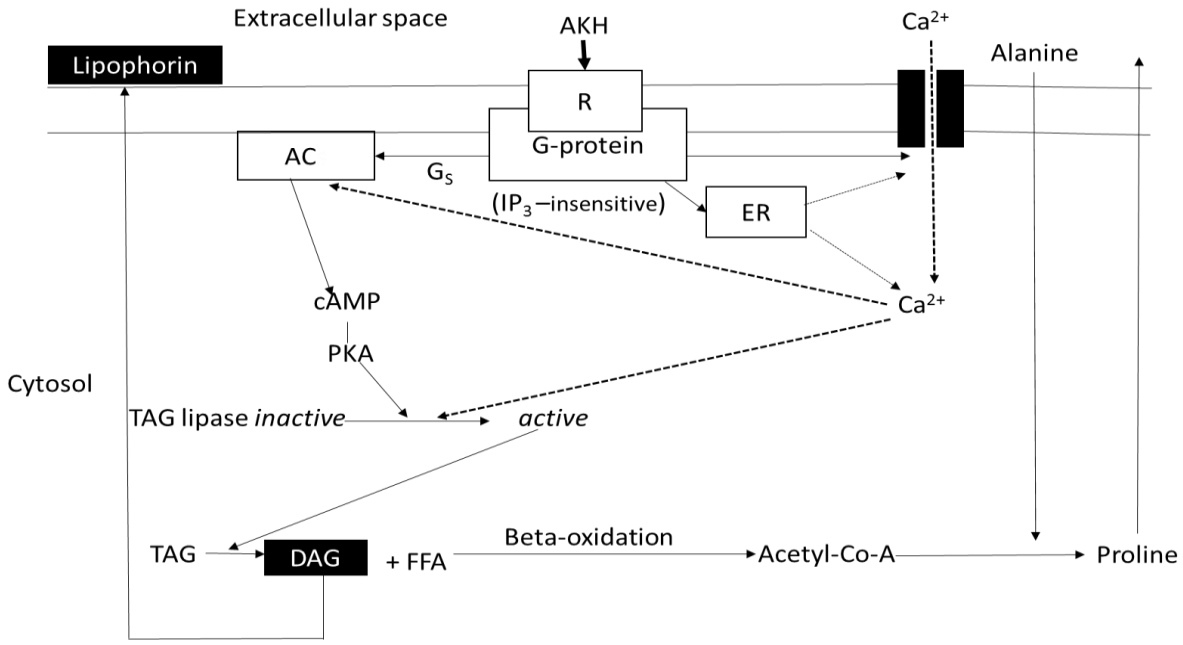

Figure 1.3. Signal transduction of the mobilisation of the Diacylglycerol/Proline signal caused by AKHs in the fat body of *L. migratoria*, *M. sexta* and *Pachnoda sinuata*. Abbreviations: co-enzyme A (CoA); DAG, diacylglycerol (DAG) and triacylglycerol (TAG). White letters on a black background indicate the pathway in *L. migratoria* only (taken from Gäde & Auerswald, 2003).

1.5. Neuropeptide regulation of circulation

In most invertebrates, including insects, the circulatory system is open type, meaning the body fluid (haemolymph) circulates freely in the body cavity, which is unlike vertebrates where the fluid travels in a network of vessels (Willmer *et al.*, 2005; Gullan & Cranston, 2014; Brooker *et al.*, 2014). Insect circulation is facilitated by the dorsal vessel, which is a longitudinal pulsatile tube that runs alongside nearly the entire length of the insect dorsal part (Gullan & Cranston, 2010). This vessel consists of the unsegmented aorta (located in the thorax and head) and a segmented heart (located in the abdomen). The whole dorsal vessel is often referred to as the insect heart (Gullan & Cranston, 2014). It is composed of one layer of myocardial cells and supported by alary muscles. In certain insects, such as *Rhodnius prolixus* and *B. extradentatum*, it is reported that the alary muscles are found mostly attached to the heart and a few on the aorta (Chiang *et al.*, 1990; Ejaz & Lange, 2008). The vessel contains openings called ostia that allow haemolymph to flow through it (Foottit & Adler, 2009; Gullan & Cranston, 2014). The dorsal vessel is innervated with cardiac nerve cords, which, together with cardiac neurons and intrinsic neurosecretory cells, make up the cardio-regulatory system. The system is tightly regulated by various neuropeptides, neurotransmitters and other factors (reviewed by Chowański *et al.*, 2016).

Proctolin, the first insect neuropeptide to be isolated and characterised (Brown & Starratt, 1975), is well known for its myotropic effects in various insects, including cardio-acceleratory effects (see reviews Orchard *et al.*, 1989; Konopińska & Rosiński, 1999; Chowański *et al.*, 2016). Two members of the AKH/RPCH family (Peram CAH-I and -II) were first recognised for their cardio-stimulatory activities in *P. americana* then later for their hypertrehalosaemic/adipokinetic effects in various insects (Scarborough *et al.*, 1984). Subsequently, various AKH/RPCH peptides and other stimuli, including the crustaceans

cardioactive peptide (CCAP), octopamine, dopamine and serotonin, were found to be capable of influencing the heart by either stimulating or inhibiting it (Lehman *et al.*, 1993; Chiang *et al.*, 1992; Dulcis *et al.*, 2005; Ejaz & Lange, 2008; Malik *et al.*, 2012). The influence of various substances on the insect heart has been studied using a variety of *in vivo* and semi-isolated biological assays. For instance, the heart beat rate of *B. extradentatum* was monitored by using electrodes inserted through the dorsal cuticle and then attached to the impedance converter (Ejaz & Lange, 2008). Some studies on insects such as *P. americana* monitored the heart beat rate by doing visual counts *in vivo* under a microscope (Baumann *et al.*, 1990). The majority of studies investigate the responses of the exposed heart with internal organs removed and the abdomen isolated from the rest of the body (Baumann *et al.,* 1990; Chaing *et al.*, 1992; Lehman *et al.*, 1993; Ejaz & Lange, 2008; Malik *et al.*, 2012; Patel *et al.*, 2014). The intensity of the influence of the stimuli on the heart varies from insect to insect, which is likely because of different experimental procedures (Chowański *et al.*, 2016).

1.6. Structure-activity studies of AKHs

The issue of the binding efficacy of AKHs in insects has been addressed mainly using *in vivo* biological assays and *in vitro* G-protein coupled receptor (GPCR) assays, testing ligands of different structures. This is done to address the question of which parts of the AKH structure are important for peptide-receptor interaction. The *in vivo* biological assays indirectly investigate the ability of an AKH to bind to its receptor by analysing the biological effects caused by various ligands in insects. The ligands could either be bioanalogues or synthetic analogues (Gäde *et al.*, 1997). Target insects are subjected to ligands of interest and the biological responses are assessed. Most these biological responses are the elevation of carbohydrates or, lipids in the haemolymph of insects (Gäde, 1986 , 1990; Hayes & Keeley,

1990; Ziegler *et al.*, 1991, 1998; Gäde, 1992; Gade, 1993; Poulos *et al.*, 1994; Gäde & Hayes, 1995; Goldsworthy *et al.*, 1997; Marco & Gäde, 2015). In a number of cases, the biological response assessed is the increase in heart beat rate of insects (Baumann *et al.*, 1990; Malik *et al*., 2012).

For *in vitro* GPCR assays, the AKH receptors are cloned and expressed to directly investigate the structural requirements of a peptide for receptor activation. These assays are mostly performed for insects where the whole genome is known. In the insect *B. mori*, which contains two AKHs (Bommo-AKH and Manse-AKH; Gäde *et al.*, 2008) and one AKH receptor (Staubli *et al.*, 2002; Zhu *et al.*, 2009), dose-response curves for Manse-AKH and Helze-AKH (another lepidopteran AKH) were constructed using the *in vitro* GPCR assay (Staubli *et al.*, 2002). The *in vitro* GPCR assay with various systematically modified analogues of tested AKH ligands has, so far, only being carried out with AKH receptors of dipteran insects (*D. melanogaster, A. gambiae* and *Glossina morsitans morsitans*) (Caers *et al.*, 2012, 2016). These structure-activity studies assessed the binding of a ligand to the *in vitro*-expressed AKH receptor. In general, the results of these *in vitro* assays agree with those generated by *in vivo* biological assays.

In general, it has been deduced from these structure-activity studies that: (1) conserved aromatic amino acids at position 4 and 8 are important for receptor-peptide interactions, (2) the blocked termini (pGlu at the N-terminal and amide at the C-terminal) are not only needed for peptide protection, but are also important for peptide- receptor interaction, and (3) insects that have two or more endogenous AKHs, such as *P. americana*, *L. migratoria* and *Hippotion eson*, seem to respond to many more analogues compared to insects with a single endogenous AKH, such as *Blaberus discoidalis.*

1.7. Scope and aims of this study

The stick insect, *Carausius morosus* (Fig. 1.4.), belongs to the family Phasmatidae (order Phasmatodea). This insect is so-called because it resemble sticks and is native to India, but is now invasive in several countries, including South Africa (Brock, 2000). The spread of *C. morosus* outside India is believed to be, primarily due to unmonitored pet trade and likely due to careless disposal of eggs (Picker & Griffiths, 2011). The species is parthenogenetic (i.e. females reproduce without mating), and polyphagous (Brock, 2000; Picker & Griffiths, 2011) hence, the Indian stick insect is an easily reared pet or study object. Despite pet status and research interest in this stick insect, it has been reported that *C. morosus* has reached the proportion of garden pest in certain countries, such as the United States of America (Picker & Griffiths, 2011). Far more severe agricultural and medical insect pests thrive in various countries worldwide, and there is a research drive towards the specific control of pest species without harming the beneficial insects, through the development of hormone-like compounds that can be used in specific drug design to act as targeted pesticides. In order for such a pesticide to function successfully, target specificity is essential.

It is with this in mind, that the stick insect *C. morosus* has been selected for examining ligand-receptor specificity with a focus on neuropeptides belonging to the AKH/RPCH family. It is hoped that the study will contribute to the design of peptidomimetics that will act to disrupt specific biological processes in target insects.

Carausius morosus produces two members of the AKH/RPCH family in its corpora cardiaca, known as hypertrehalosaemic hormone-I and -II (Carmo-HrTH-I and –II, Table 1.1; Gäde, 1985; Gäde *et al*., 1992). Both of these HrTHs are decapeptides, and they differ only at position 8, where the tryptophan of Carmo-HrTH-I is C-mannosylated. These peptides are so far unique to Phasmatodea (Gäde, 2009) and, therefore, present an ideal test-case to study

specificity of the receptor for Carmo-HrTH. Hence, the objective of the current study was to determine whether other members of the AKH/RPCH family produced in insect species outside the Phasmatodea order, but structurally similar to the Carmo-HrTHs - could function in stick insects. This information would help to establish whether stick insects would be affected by drugs (peptidomimetics based on the peptide structure of other AKH/RPCHs) that are meant to target other pest species.

The specific aims of the current study were: (1) to test a range of naturally-occurring AKHs in *C. morosus* for functional activity, and (2) to determine which structural features of Carmo-HrTH-II is important in interacting with the *C. morosus* receptor for HrTH. Carmo-HrTH-I was not selected as lead peptide since the mannosylation of Trp^{8} is an unusual post-translational modification in the AKH/RPCH peptide family, and pilot studies showed that Carmo-HrTH-II is equally active in *C. morosus*.

To achieve the aims of the study, three sets of peptides were investigated (see Table 1.2 for primary sequences). The first set consisted of N- and C-terminally modified analogues. Carmo-HrTH-II is blocked at the N- and C-terminus by a pyroglutamic acid **(**pGlu) and an amide, respectively. Hence, this set was designed to evaluate the importance of these blocking residues. The set consisted of analogues of Carmo-HrTH-II without an amide (i.e. as non-amidated free acid), without pGlu (thus, as an unblocked nonapeptide), and with pGlu replaced by an N-acetyl-alanine residue ([N-Ac-Ala^{1}]-Carmo-HrTH-II, thus it remains a blocked peptide). The removal of an amide has the additional effect of making the analogue negative, whereas the native peptide is neutral (Gäde & Hayes, 1995). The removal of the pGlu, on the other hand, makes the analogue shorter than the native peptide, while the replacement of pGlu with N-Ac-Ala keeps the peptide blocked at the N-terminus, but

changes its flexibility (thus, *cyclic* vs *acyclic* side chain) and the hydrogen bonding potential (thus, *trans* vs. *cis*) of the N-terminus (Gäde & Hayes, 1995).

The second set consisted of three naturally-occurring AKH octapeptides: Aedae-AKH, Peram-CAH-II, and Pyrap-AKH, that were first isolated from a mosquito, cockroach and bug, respectively. Peram-CAH-II is identical to Carmo-HrTH-II, except that it lacks the two additional amino acids present at the C-terminal end of the stick insect HrTH. Aedae-AKH and Pyrap-AKH differ from Peram-CAH-II only at one position: 7 and 3, respectively (see Table 1.2 for primary sequences). These peptides were assessed in order to determine whether the HrTH receptor of *C. morosus* has an affinity for shorter peptides, since the minimum chain length of AKH/RPCH family peptides is 8 amino acids.

The third set consisted of naturally-occurring decapeptide AKHs and systematically altered analogues based on Carmo-HrTH-II as lead peptide. The selected decapeptide AKHs have a single or double amino acid replacement in comparison with Carmo-HrTH-II, and were first isolated from locusts and large grasshoppers (see Table 1.2 for primary sequences). The specificity of the Carmo-HrTH receptor would, thus, be revealed from interactions with this set of decapeptides. The systematically altered analogue series has one amino acid replaced by an Ala residue, starting with Ala^{2} and ending with Ala^{10} (Table 1.2). The substitution with an Ala residue has the effect of removing the side chain of the substituted amino acid without disrupting the peptide backbone (Gäde & Hayes, 1995). This set was specifically designed to determine which amino acids and side chains are crucial for the HrTH peptide-receptor interaction in *C. morosus*.

The functional performance of the peptides under investigation was assessed in *C. morosus* via two *in vivo* biological assays: the carbohydrate-mobilising bioassay was used to measure the increase of total carbohydrates in the haemolymph (i.e. hypertrehalosaemia), while the semi-exposed heart assay measures a change in heart beat rate (i.e. contraction of the heart – myotropy).

Figure 1.4. Adult *Carausius morosus* (Indian stick insect) on a flowering shrub *Coprosma repens*. The picture was taken at the colony cultured in the Biological Sciences Department of the University of Cape Town. Photo credit: Ottilie Katali.

Table 1.2. The primary sequences of the AKH bioanalogues and systematically altered analogues of Carmo-HrTH-II used in the present study.

Peptide name	Primary sequence										
Carmo-HrTH-I	pGlu	Leu	Thr	Phe	Thr	Pro	Asn	**Trp***	Gly	Thr	amide
Carmo-HrTH-II	pGlu	Leu	Thr	Phe	Thr	Pro	Asn	Trp	Gly	Thr	amide
Aedae-AKH	pGlu	Leu	Thr	Phe	Thr	Pro	**Ser**	Trp	amide		
Peram-CAH-II	pGlu	Leu	Thr	Phe	Thr	Pro	Asn	Trp	amide		
Pyrap-AKH	pGlu	Leu	**Asn**	Phe	Thr	Pro	Asn	Trp	amide		
Locmi-AKH-I	pGlu	Leu	**Asn**	Phe	Thr	Pro	Asn	Trp	Gly	Thr	amide
Phyle-CC	pGlu	Leu	Thr	Phe	Thr	Pro	Asn	Trp	Gly	**Ser**	amide
Phymo-AKH	pGlu	Leu	**Asn**	Phe	Thr	Pro	Asn	Trp	Gly	**Ser**	amide
Rommi-CC	pGlu	**Val**	**Asn**	Phe	Thr	Pro	Asn	Trp	Gly	Thr	amide

Peptide name	Primary sequence										
Carmo-HrTH-II	pGlu	Leu	Thr	Phe	Thr	Pro	Asn	Trp	Gly	Thr	amide
[Trp-OH]	pGlu	Leu	Thr	Phe	Thr	Pro	Asn	Trp	Gly	Thr	**OH**
[H-Leu]	**H-**	Leu	Thr	Phe	Thr	Pro	Asn	Trp	Gly	Thr	amide

Peptide name	Primary sequence										
Carmo-HrTH-II	pGlu	Leu	Thr	Phe	Thr	Pro	Asn	Trp	Gly	Thr	amide
[N-Ac-Ala^{1}]	**[N-Ac-Ala]**	Leu	Thr	Phe	Thr	Pro	Asn	Trp	Gly	Thr	amide
[Ala^{2}]	pGlu	**Ala**	Thr	Phe	Thr	Pro	Asn	Trp	Gly	Thr	amide
[Ala^{3}]	pGlu	Leu	**Ala**	Phe	Thr	Pro	Asn	Trp	Gly	Thr	amide
[Ala^{4}]	pGlu	Leu	Thr	**Ala**	Thr	Pro	Asn	Trp	Gly	Thr	amide
[Ala^{5}]	pGlu	Leu	Thr	Phe	**Ala**	Pro	Asn	Trp	Gly	Thr	amide
[Ala^{6}]	pGlu	Leu	Thr	Phe	Thr	**Ala**	Asn	Trp	Gly	Thr	amide
[Ala^{7}]	pGlu	Leu	Thr	Phe	Thr	Pro	**Ala**	Trp	Gly	Thr	amide
[Ala^{8}]	pGlu	Leu	Thr	Phe	Thr	Pro	Asn	**Ala**	Gly	Thr	amide
[Ala^{9}]	pGlu	Leu	Thr	Phe	Thr	Pro	Asn	Trp	**Ala**	Thr	amide
[Ala^{10}]	pGlu	Leu	Thr	Phe	Thr	Pro	Asn	Trp	Gly	**Ala**	amide

Residues that differed from Carmo-HrTH-II are indicated in bold and underlined. The asterisk indicates that the Trp residue is C-mannosylated.

2. Chapter two: Materials and methods

2.1. Experimental insects: *Carausius morosus*

2.1.1. Maintenance of the insect colony

Indian stick insects (*Carausius morosus),* specifically 6th instar nymphs and adults, were obtained from a colony cultured in the insect room of the Department of Biological Sciences, University of Cape Town. This colony was started with insects initially collected from ivy plants by Dr Heather Marco and Prof Gerd Gäde in 1996 in Cape Town. In the insectary, adult insects were reared from eggs in a controlled environment. Environmental conditions were 25 ± 2 ^{o}C ambient temperature, about 65 % relative humidity and a light regime of 12h light: 12h dark. Humidity was maintained by filling open trays and bottles with water.

Eggs were kept in glass cages (L= 22 cm, W= 15 cm and H= 22 cm) lined with newspaper and fitted with a mesh lid. The egg cages were checked daily for newly hatched nymphs. These nymphs were removed and placed in a separate glass cage of similar size containing food, branches of ivy (*Hedera helix)*. Older nymphs were transferred to mesh wire cages (L= 26 cm, W= 40 cm and H= 44 cm). Once reached the adult stage, they were transferred to bigger mesh wire cages (L= 80 cm, W= 50 cm and H= 80 cm) containing branches of mirror bush (*Coprosma repens*) and allowed to lay eggs. The eggs were collected on a monthly basis and allowed to hatch to re-stock the colony. The cut ends of plant branches were placed into bottles of water. The branches were replaced with the fresh ones at least twice a week.

2.1.2. Monitoring of the developmental stages

The study worked with specific age groups of 6th instar nymphs and adults. In order to ensure the right ages were used, the study had to first determine the different instars of the stick insects. These was achieved by randomly selecting 50 freshly hatched nymphs from the

entire colony. These freshly hatched stick insects were gently marked on the thorax using a permanent marker pen and their body lengths were measured using a ruler. They were then kept in a separate cage under the same conditions as the entire insect colony. This sample was monitored on a daily basis. Once each insect moulted, its body length was measured and marked again. The measuring and marking were carried on after every moult until the insects became adults. After determine all the different instars of this stick insect, the daily monitoring and the markings were still done for the 5th instar nymphs and older of the entire colony. Thus, all the nymphs were marked and kept in one cage as they reached the 5th instar stage. Once they moulted to the 6th instar, they were marked and transferred to a separate cage on the daily basis. These 6th instars were marked according to the date in order to keep track of their ages for use in bioassays. Those that were not used for bioassays at this stage were allowed to moult to adults. The adults were then allowed to lay eggs and some used in bioassays.

2.2. Extraction of hypertrehalosaemic peptides from the corpora cardiaca (CC) of *C. morosus*

Natural hypertrehalosaemic hormones, Carmo-HrTH-I and -II, were obtained from the CC of *C. morosus*. These CC were dissected from adult *C. morosus*. For anaesthetisation, insects were kept on ice for a few minutes prior to the dissection. Head capsules were then opened using a razor blade to expose the CC. The opened head capsules were pinned into a dissecting dish. Under a dissecting microscope (Nikon, Japan) with an external light source (Intralux 5000, Volpi Manufacturing, USA), the CC were removed using fine scissors and forceps and then stored in a 1.5 ml Eppendorf tube containing 300 µl of 80% methanol (Merck, South Africa). These aliquots of 5 to 15 dissected CC in methanol were stored at -20 ^{o}C prior to the extraction of peptides.

Peptides were extracted based on the methods by Gäde *et al.* (1984) with several modifications. To open the cells, the CC stored in methanol were sonicated for 30 s using an ultrasonic cell disruptor B-30 (Branson Sonic Power Co., USA). Eppendorf tubes were kept on ice during the disruption process to avoid peptide degradation due to heat produced by the sonifier. The homogenised solution was centrifuged for 4 min at RCF of 11 000×g (Biofuge 15, Heraeus Sepatech, Germany) at room temperature. The supernatant, containing peptides, was transferred to a new Eppendorf tube and dried in a speed vacuum concentrator (Bachofer, Germany). Dried crude extracts of CC were stored at -20 ^{o}C until reconstituted either in acetonitrile or distilled water for fractionation by reversed phase-high performance liquid chromatography (RP-HPLC) or for use in biological assays, respectively.

2.3. Isolation of Carmo-HrTH-I and -II using RP-HPLC

Carmo-HrTH-I and -II were isolated from the dried CC extract of *C. morosus* by RP-HPLC according to the modified method of Gäde (1985). Dried CC extracts were reconstituted in 15% acetonitrile containing 0.10% triflouroacetic acid (TFA) and applied to a Nucleosil 100 C-18 column (dimensions: 250 × 4 mm; 5µm particles) for RP-HPLC, monitored by a FP-920 intelligent fluorescence detector (Jasco, Japan) (excitation 276 nm, emission 350 nm and attenuation of 8), which targets the Trp at position 8. The RP-HPLC system consisted of the following components: a model 302 piston pump with 5.Sc pump head, model 305 with 5.S pump head, manometric model 802 and a model 811 dynamic mixer (all Gilson, Inc.). The system also consists of a DG-1580-54 degasser (Jasco, Japan) and it is connected to a data chart recorder model BD40 (Kipp and Zonen, Netherlands). The sample injector valve is a model 7125 with 500 µl sample loop (Rheodyne, Germany). The column was developed with a linear gradient of 0.11% TFA in water (solvent A) and 0.10% TFA in 60% acetonitrile

(solvent B) from 43% to 53% B in 20 min at a flow rate of 1 ml /min. Peak fractions were collected manually into Eppendorf tubes during the separation process, dried using the speed vacuum concentrator, and stored at -20 ^{o}C until when reconstituted in distilled water or saline for use in biological assays.

2.4. Synthetic naturally-occurring adipokinetic peptides and systematically altered analogues

The primary sequences of the synthetic peptides are shown in Table 1.2. Carmo-HrTH-II, Locmi-AKH-I and Peram-CAH-I were synthesised by Peninsula Laboratories (USA). Carmo-HrTH-II minus amide (= free acid), Carmo-HrTH-II minus pGlu and Phymo-AKH were synthesised by Dr. S. Kyin (Biotechnology Centre, University of Illinois, Urbana-Champaign, USA). Phyle-CC and Pyrap-AKH were synthesised by Dr. R. Kellner (Merck KGaA, Germany), and Aedae-AKH was made by Genscript Corporation (USA). Analogues of Carmo-HrTH-II with single amino acids replaced with Ala were purchased from Pepmic Co. Ltd (China). Solutions were made by dissolving 1 mg of each peptide in 1 ml of the RP-HPLC solvent B (60 % acetonitrile and 0.10 % TFA). Solutions were diluted to 5 pmol/µl using distilled water, and the concentrations of the peptides were verified by RP-HPLC. These solutions were then stored at -20 ^{o}C in aliquots of 100 µl until further dilutions were made using distilled water prior to the experiments.

2.5. Proctolin, crustacean cardioactive peptide (CCAP) and octopamine

Proctolin (Arg-Tyr-Leu-Pro-Thr) (Alfa Aesar, USA) and CCAP (Pro-Phe-Cys-Asn-Ala-Phe-Tyr-Gly-Cys-NH_2) (Bachem AG, Switzerland) were made up as stock solutions of 10^{-3} M for Proctolin and 10^{-2} M for CCAP using distilled water. Stock solutions were stored as aliquots of 10 µl at -20 ^{o}C until experimental dilutions were made using saline. The solutions of DL-

Octopamine hydrochloride (Sigma, South Africa) were prepared using saline on the day of experimentation.

2.6. Stick insect physiological saline

The saline used was prepared based on the recipe by Ejaz & Lange (2008). Chemicals in Table 2.1 were weighed on an analytical balance (Ohaus Adventurer, USA) and dissolved in 300 ml of distilled water. The pH was adjusted to 6.6 with drops of 0.1 M sodium hydroxide (Univar, South Africa). Sodium hydroxide was mixed in using a magnetic stirrer (Heidolph, Germany) and pH was measured using a pH meter (Orion, USA). . The saline was stored at 4 ^{o}C and was always allowed to warm up to 26 ± 2 ^{o}C prior to biological assays.

Table 2.1. The composition of stick insect physiological saline (volume 300 ml).

Chemical name	Mass (g)	Molarity (mM)
NaCl (Univar, South Africa)	0.263	15
KCl (Univar, South Africa)	0.402	18
HEPES (Sigma, South Africa)	0.143	2
$MgCl_2.6H_2O$ (Merck, South Africa)	3.035	50
$CaCl_2.2H_2O$ (Univar, South Africa	0.332	7.5
Anhydrous glucose (Univar, South Africa)	9.945	184

2.7. Measuring of carbohydrates in haemolymph

Haemolymph was taken either from 6th instar nymphs or adult *C. morosus* at 25 ± 2^{o}C. For the carbohydrate-mobilising bioassay, insects (age and sample size specified in Results) were ligated at the neck using a thin thread. Ligated insects were then covered individually with a plastic funnel and left for about 2 hours before haemolymph collection. To obtain the haemolymph from the insect, the soft membrane at the base of a hind leg was pierced with

a pin. Using a 1 µl glass capillary (Hirschman Laborgeräte, Germany), 1 µl of haemolymph was collected by means of capillary force and immediately blown into a glass test tube containing 100 µl of concentrated H_2SO_4 (Kimix, South Africa). Then, the abdomen of the insect was injected with 10 µl of test solution, using a 25 µl Hamilton syringe. These insects were left for 90 minutes before a second 1 µl sample of haemolymph was collected. This assay was carried out according to Gäde & Lohr (1982). For the determination of the normal concentration of carbohydrates in the haemolymph of *C. morosus*, haemolymph was collected from live insects that had not been injected with anything.

The haemolymph samples were analysed for carbohydrates (i.e., anthrone-positive material) after Spik & Montreuil (1964; text translated by G. Gäde). The anthrone reagent was prepared by adding 0.6 g of anthrone powder (Sigma-Aldrich, USA) to 300 ml of concentrated H_2SO_4 (Kimix, South Africa) in a 500 ml dark glass bottle. The solution was stirred with a magnetic stirrer (Heidolph, Germany) until the anthrone was dissolved. The bottle was then placed in a bucket containing ice, and 150 ml of distilled water was added drop by drop to the acid using a volumetric burette. The anthrone reagent was stored in the dark at room temperature and was used within 3 weeks after preparation.

To the haemolymph sample in concentrated H_2SO_4, anthrone reagent (2 ml) was added and mixed using a vortex mixer (Fisons, England). Tubes were subsequently heated for 8 min in a Thermolyne dri-bath (Thermo Scientific, USA) at 100 ^{o}C and cooled in cold tap water for 5 min. After cooling, tubes were kept in the dark at room temperature for 30 min. The optical densities of the solutions were then measured using a LKB Biochrom Ultrospec 4050 UV/Vis Spectrophotometer (Biochrom Ltd, United Kingdom) at a wavelength of 585 nm in a glass cuvette against a reagent blank.

The measured absorbance values were translated into the concentration of carbohydrates (mg/ml) using a glucose standard curve. To construct this curve, 160 mg of glucose powder (Analar, BDH Chemicals Ltd., England) was dissolved in 1 ml of distilled water to give an initial concentration of 160 mg/ml. This concentration was further diluted to 80, 40, 20, 10, 5 and 2.5 mg glucose per ml of water. From each of these solutions, 1 µl was collected by means of a glass capillary (Hirschman Laborgeräte, Germany) and blown into a glass test tube containing 100 µl of concentrated H_2SO_4 (Kimix, South Africa). Solutions were then analysed for anthrone-positive material as described earlier. The absorbances were plotted against their respective glucose concentrations to generate the standard curve.

2.8. Semi-exposed heart bioassay

The semi-exposed heart bioassays were carried out based on previously described assays (Baumann *et al.*, 1990; Chiang *et al.*, 1992) at 25 ± 2 ^{o}C. Adult stick insects between 1 and 2 months old were used. First, the legs were cut off the body followed by a ventral incision of the abdomen. In case of decapitated or ligated insects, the decapitation/neck- ligated was done prior to cutting off the legs. The preparation was then pinned open on the dissecting dish using minutien pins (dorsal part down) and a few drops of saline were added to the exposed part. The internal organs (i.e., alimentary canal, ovaries and immature eggs) were carefully shifted out of the exposed part to the side of the body but still remained attached (see Fig. 2.1). Thereafter, haemolymph from the preparation was replaced with saline. These saline continued to be replaced with fresh saline (150 µl) every 10 to 15 minutes. After every fluid replacement, the heart beat rate was counted by manually counting the beats in one minute under a dissecting microscope (Wild Heerbrugg, Switzerland; magnification of X12). The replacement of saline was done until the heart beat rate stabilised (i.e., when similar heart beat counts were obtained in a subsequent count).

Once the heart stabilised, saline was replaced with 150 µl of the test solution. Thereafter, 8 visual counts (i.e., beats per minute) were done, subsequently within 15 minutes after the application of test solutions. Between the applications of test solutions, the preparations were washed several times with repeated changes of saline until the heart stabilised again.

Heart beat rates under 150 µl of normal saline served as controls. The change in heart beat rate rate after the application of the test solution was calculated as an average of the first four counts and was compared to the average heart beat rates counted in saline before the application of the test solution. This comparison of change in heart beat rates was done for the all the peptides tested. For octopamine, all eight counts were used for comparison to the average heart beat rate counted in saline before application.

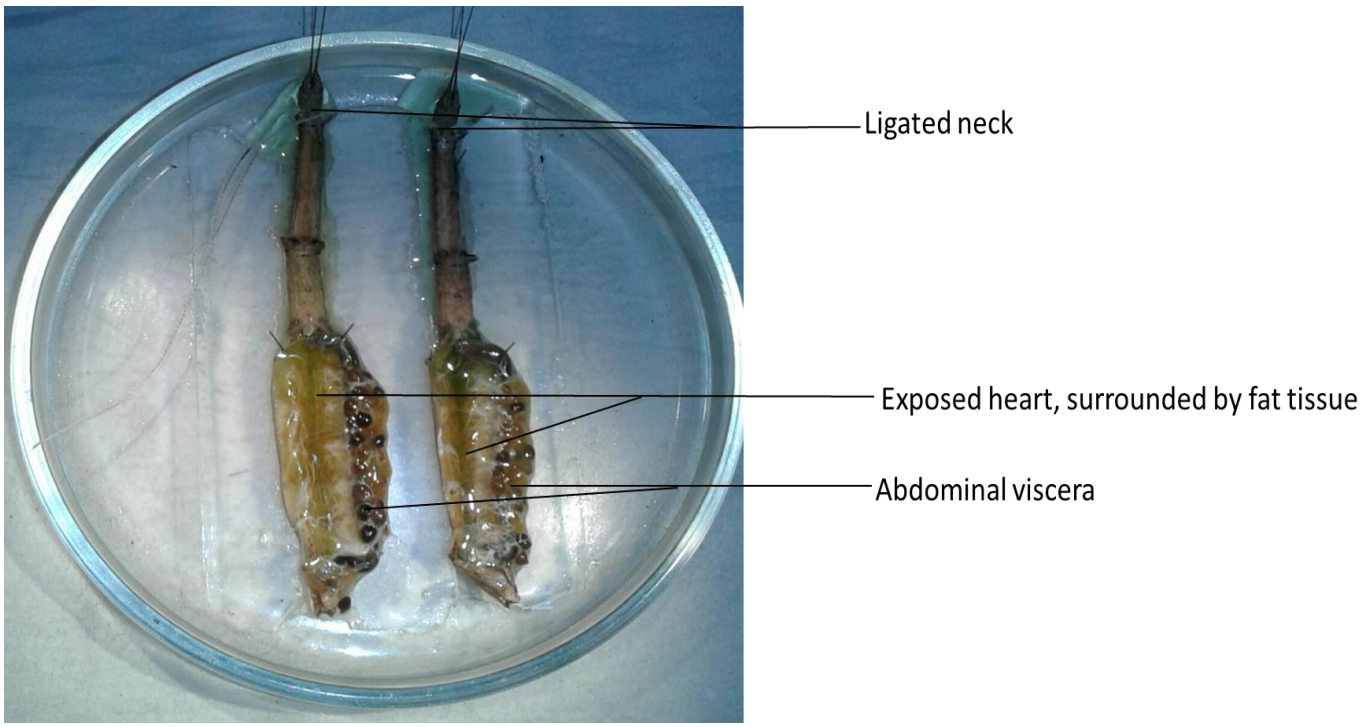

Figure 2.1. Preparations of two semi-exposed hearts of the neck-ligated adult *C. morosus*.

2.9. Statistical analyses

The concentration of carbohydrates in the haemolymph of different age groups of *C. morosus* was analysed using a one-way analysis of variance (ANOVA) followed by Scheffe's multiple comparison test (IBM SPSS Statistics 23 software, IBM corporation, United States). For the carbohydrate-mobilising and semi-exposed heart assays, a Student paired t-test was used to compare the concentrations of carbohydrates in the haemolymph as well as the heart beat rates of *C. morosus* before and after the subjection to the test solution. The one-way ANOVA followed by Scheffe's multiple comparison test was again used to compare the hypertrehalosaemic and cardio-stimulatory effects among *C. morosus* tested with different substances. Differences were considered significant at $p < 0.05$ for all the tests.

3. Chapter three: Results

3.1. Life cycle of the stick insects

The present study targeted to work with the 6th instar nymphs and adults of *Carausius morosus*. Hence, insects were monitored on the daily basis to determine all the developmental stages of these insects and thereafter focused on the age groups of interest. Daily monitoring observations revealed that the freshly hatched stick insects (11 mm) moult 6 times before becoming adults (>70mm). It was also noticed that eggs hatch after 2 months. Once hatched, the freshly hatched nymphs take about 3 months before becoming adults. Adults start laying eggs at about 2 weeks old and live for about 3 to 4 months. The larval stage durations vary from about 1.5 to 3 weeks. The body lengths of all the developmental stages of *C. morosus* are shown in Fig. 3.1.

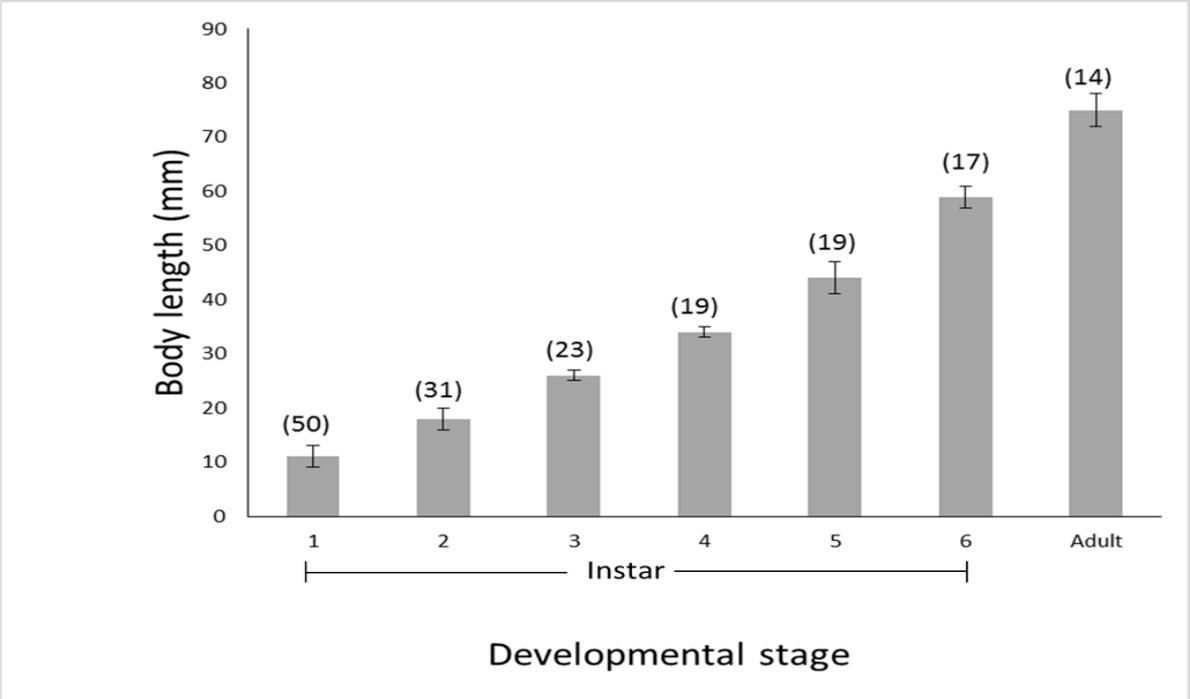

Figure 3.1.The average body length of *C. morosus* at each larval stage and adult. Each bar represents the mean ± SD, and the sample size (shown in brackets).

3.2. Purification of Carmo-HrTH-I and -II from the corpora cardiaca (CC) extract of C. morosus

The two hypertrehalosaemic hormones (HrTHs) of *C. morosus* can be isolated successfully from its CC (Gäde, 1985). Hence, the present study attempted to isolate these hormones from the CC of this species for use in bioassays. As depicted in Fig. 3.2., the fluorescent trace showed two distinct peaks that eluted at about 7 and 11 min. According to Gäde (1985), these peaks are the Carmo-HrTH-I and -II, respectively, which have hypertrehalosaemic activity in the ligated stick insect (Table 3.1).

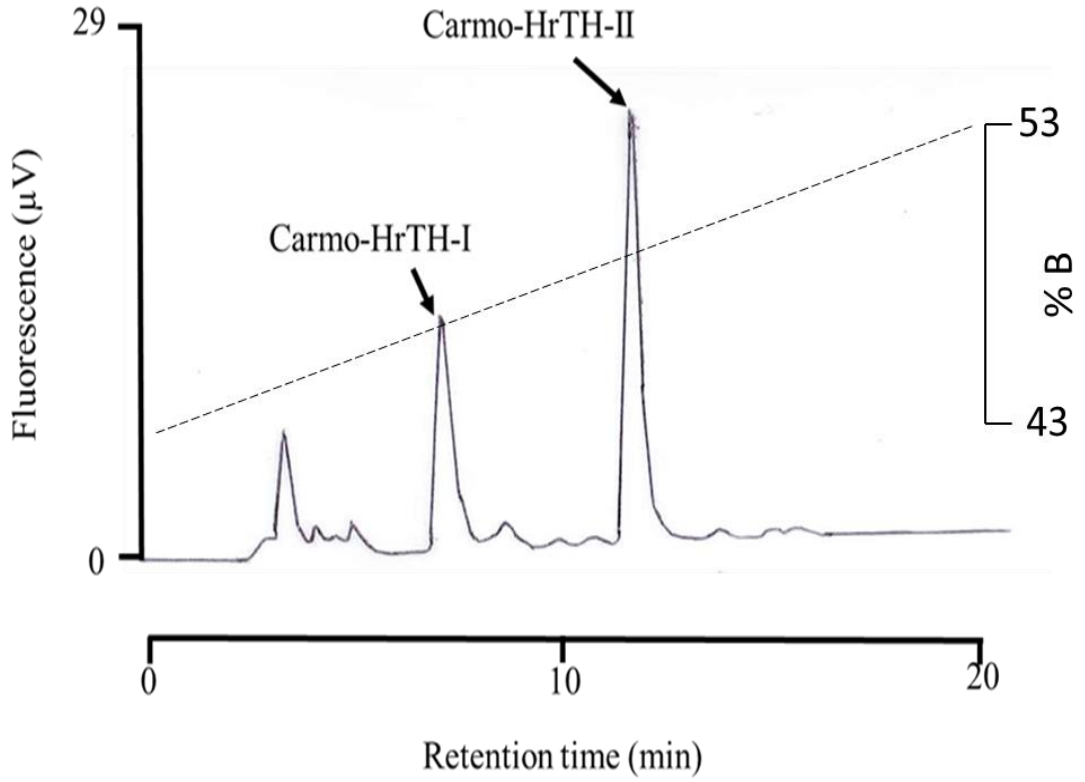

Figure 3.2. Typical fluorescence (excitation 276 nm; emission 350 nm) profile of a methanolic crude extract from *C. morosus* corpora cardiaca. Methanolic extract was prepared as described in Chapter 2: the dried material was reconstituted in 15% acetonitrile containing 0.10% TFA and applied to a Nucleosil C18 column for RP-HPLC. Aqueous solvent A was water with 0.11% TFA. Organic solvent B was 60% acetonitrile containing 0.10% TFA. The two solvents were used to apply a linear gradient from 43% to 53% solvent B in 20 min at a flow rate of 1 ml/min. Carmo-HrTH-I and -II were collected manually, dried *in vacuo* and reconstituted in water or saline prior to the biological assays.

3.3. Carbohydrate-mobilising bioassays

3.3.1. Determination of the ideal developmental stage of stick insects for experiments

Maximum hypertrehalosaemic response of *C. morosus* was observed in neck-ligated 6th instar nymphs that were about to undergo ecdysis (Gäde & Lohr, 1982). Ligation itself did not have any impact on the concentration of carbohydrates, but it restricted the flow of haemolymph around the abdomen. This stage of the 6th instars about to moult is when the carbohydrates in the haemolymph were high (Lohr & Gäde, 1983). Therefore, the current study targeted to work with the same developmental stage.

3.3.1.1. Age-related changes in the concentration of carbohydrates in the haemolymph

The concentration of carbohydrates in the haemolymph of *C. morosus* 6th instar nymphs and adults of different age groups was measured. This was done in order to determine the pattern of the levels of carbohydrates in the haemolymph of these age groups. As illustrated in Fig. 3.3, the concentration of carbohydrates was low (about 4 mg/ml) in 6th instar nymphs younger than 14 days old and thereafter increased to about 8 to 9 mg/ml. After ecdysis, the level of total carbohydrates decreased back to about 4 mg/ml. The one-way ANOVA revealed that there was a significant difference between the age groups ($F_{12,114}$ = 14.942, $p < 0.0001$. A post hoc Scheffe's test showed that the 6th instars aged 15 to 19 days and 1 day old-adults differed significantly ($p < 0.05$) from the younger 6th instars (≤ 14 days old) and older adults (≥ 2 days old) (Fig. 3.3).

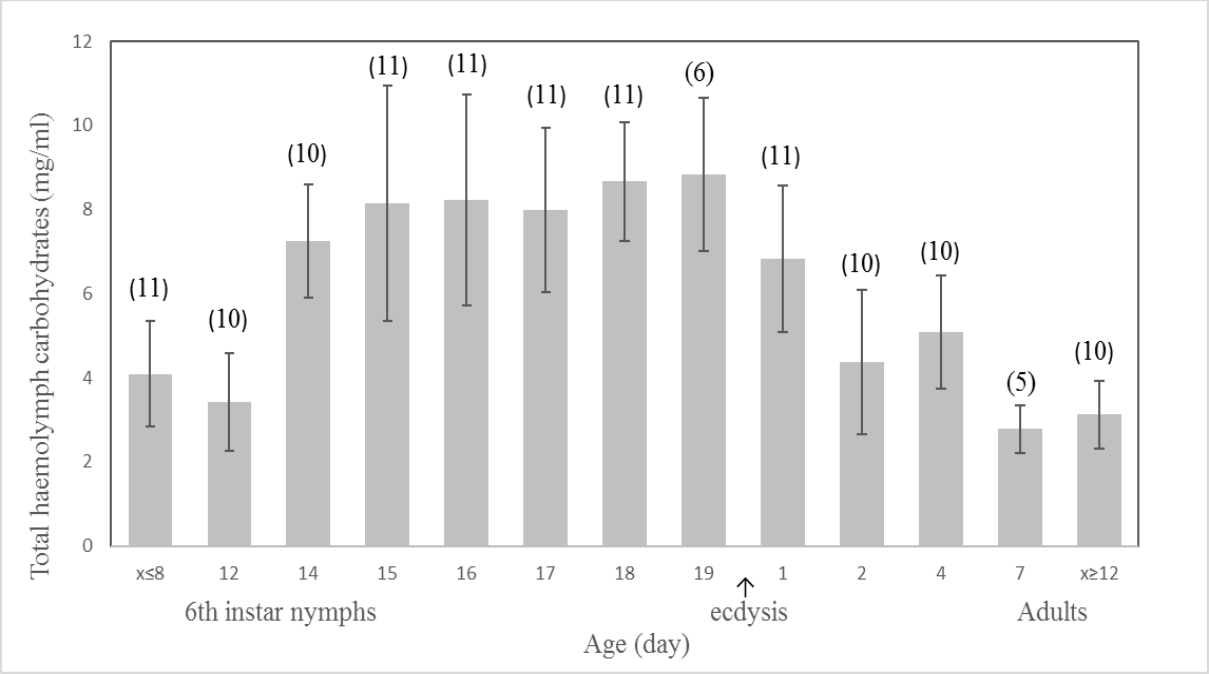

Figure 3.3. Age-related changes in the concentration of total carbohydrates in the haemolymph of *C. morosus*. Each bar represents the mean ± SD, and the sample size (shown in brackets).

3.3.1.2. Age-related response to Carmo-HrTH-II in elevating the concentration of carbohydrates in the haemolymph of C. morosus

The first series of biological assays carried out in the current study was to confirm the ability of Carmo-HrTH-II to elevate the concentration of carbohydrates in the haemolymph of *C. morosus*, as previously reported (Gäde & Lohr, 1982). It was also reported that this hypertrehalosaemic activity was only observed in neck-ligated *C. morosus*, but not in non-ligated ones (Gäde & Lohr, 1982). Therefore, these biological assays were carried out in ligated 6th instar nymphs (17 to 18 days old). Natural Carmo-HrTH-I and II (isolated from the CC of the stick insects of our colony) and synthetic Carmo-HrTH-II were tested and distilled water served as a control.

The results show that water did not elevate the level of carbohydrates in the haemolymph of the insects ($p > 0.05$) whereas the ones injected with the endogenous HrTHs increased significantly (Table 3.1). All three peptides caused an increase of about 5 mg/ml in

haemolymph carbohydrates (Table 3.1). There was no significant difference between the increases caused by these three peptides, therefore it was decided to use synthetic Carmo-HrTH-II as the lead peptide in future assays.

The effect of synthetic Carmo-HrTH-II on carbohydrate titre in 6th instar *C. morosus* nymphs (ligated) at different stages of development were tested. Results are depicted in Table 3.2. The highest response among the age groups tested was observed in ligated 6th instar nymphs that were 17 to 18 days old (injected with 20 pmol) and also the ones that were 10 - 12 days (injected with 50 pmol). A One-way ANOVA showed a significant difference between all the age groups tested ($F_{3, 36}$ = 6.518, p = 0.001). A post hoc Scheffe's test showed that the response of older 6th instar nymphs differed significantly (p = 0.014) from younger nymphs injected with the same dose (20 pmol). Also, the response of the younger nymphs injected with a higher dose (50 pmol) was not significantly different from that of the adults and older nymphs injected with 20 pmol (p > 0.05). The increase in haemolymph carbohydrates observed in older nymphs was not significantly different to the increase found in adults (p > 0.05). However, adults had a lower average increase in haemolymph carbohydrates than the older nymphs. Therefore, the 6th instar nymphs aged 17 to 18 days old were used in all future carbohydrate-mobilising assays to attain a maximum response from the animals.

Table 3.1. Effects of Carmo-HrTH-I and -II on the concentration of carbohydrates (mean ± SD) in the haemolymph of ligated 6th instar nymphs (17 to 18 days old).

Treatment (10 µl)	Haemolymph (mg/ml)				
	n	0 min	90 min	Difference	*p*
Distilled water	18	8.4 ± 1.6	8.7± 1.6	0.3±1.0	NS
Carmo-HrTH-I (natural;20 pmol)	12	8.0 ± 1.6	12.9 ± 1.9	4.9±1.1	0.00001
Carmo-HrTH-II(natural;20 pmol)	19	8.5 ± 1.8	13.1 ± 1.9	4.6±1.3	0.00001
Carmo-HrTH-II(synthetic;20 pmol)	21	8.8 ± 1.7	13.8 ± 2.4	5.0±1.3	0.00001

Student paired t-test was used to compare values at 0 min to values at 90 min; NS = not significant.

Table 3.2. The carbohydrate-mobilising effect of Carmo-HrTH-II in different developmental stage of *C. morosus*.

Developmental stage (age in days)	Haemolymph (mg/ml)			
	Dose (pmol)	0 min	90 min	Difference
6th instar (11-12)	20	3.7 ± 0.6	7.1 ± 1.1	3.4 ± 0.7
6th instar (10-12)	50	4.0 ± 0.5	9.5 ± 0.8	5.5 ± 0.8
6th instar (17-18)	20	9.4 ± 1.5	14.9 ± 1.9	5.5 ± 1.1
Adults (7)	20	4.9 ± 1.0	8.9 ± 2.6	4.0 ± 2.2

Data are presented as mean ± SD for 10 observations
A student paired t-test was used to compare values at 0 min to values at 90 min; there were significant differences ($p < 0.05$) for all age groups.

3.3.1.3. Dose-response relationship for Carmo-HrTH-II and corpora cardiaca (CC) extract of C. morosus

Carmo-HrTH-II and CC extract were used to determine the maximum response of *C. morosus* in carbohydrate-mobilising biological assays. Different doses of synthetic Carmo-HrTH-II and CC extract were tested for hypertrehalosaemic activity in ligated *C. morosus*. The *C. morosus* tested were in their 6th instar stage and were 17 to 18 days old. Both Carmo-HrTH-II (Fig. 3.4) and CC (Fig. 3.5) extracts increased the level of haemolymph carbohydrates in a dose-dependent manner. Doses of 0.04 pmol of synthetic Carmo-HrTH-II and 0.005 pairs of corpus cardiaca equivalent (pCC) of *C. morosus* were sufficient to give significant increases in haemolymph carbohydrates ($p < 0.05$).

There was a significant difference between the groups injected with different doses for both Carmo-HrTH-II ($F_{10, 105} = 30.285$, $p < 0.0005$) and the CC extracts ($F_{7, 67} = 33.680$, $p < 0.0005$,). With synthetic Carmo-HrTH-II, doses of 7.5 to 60 pmol gave an average increase of 5.2 ± 0.4 mg/ml and the post hoc Scheffe's test revealed that there was no significant difference between these doses ($p > 0.05$). Doses of 0.1 to 0.5 pCC of CC extract gave an average increase of 5.6 ± 0.6 mg/ml in the haemolymph carbohydrates and there was also no significant difference between these doses ($p > 0.05$). Strangely, a lower dose, 0.05 pCC extract, gave a higher increase (8.5 ± 1.0 mg/ml) than all the doses tested. This dose was considered an outliner because its increase differed significantly ($p > 0.05$) from all the higher CC extract doses and its increased was not achieved by any of the Carmo-HrTH-II doses either. Hence, dose of 20 pmol that showed hypertrehalosaemic activity within the common maximum range was chosen as a standard for testing the analogues of Carmo-HrTH-II.

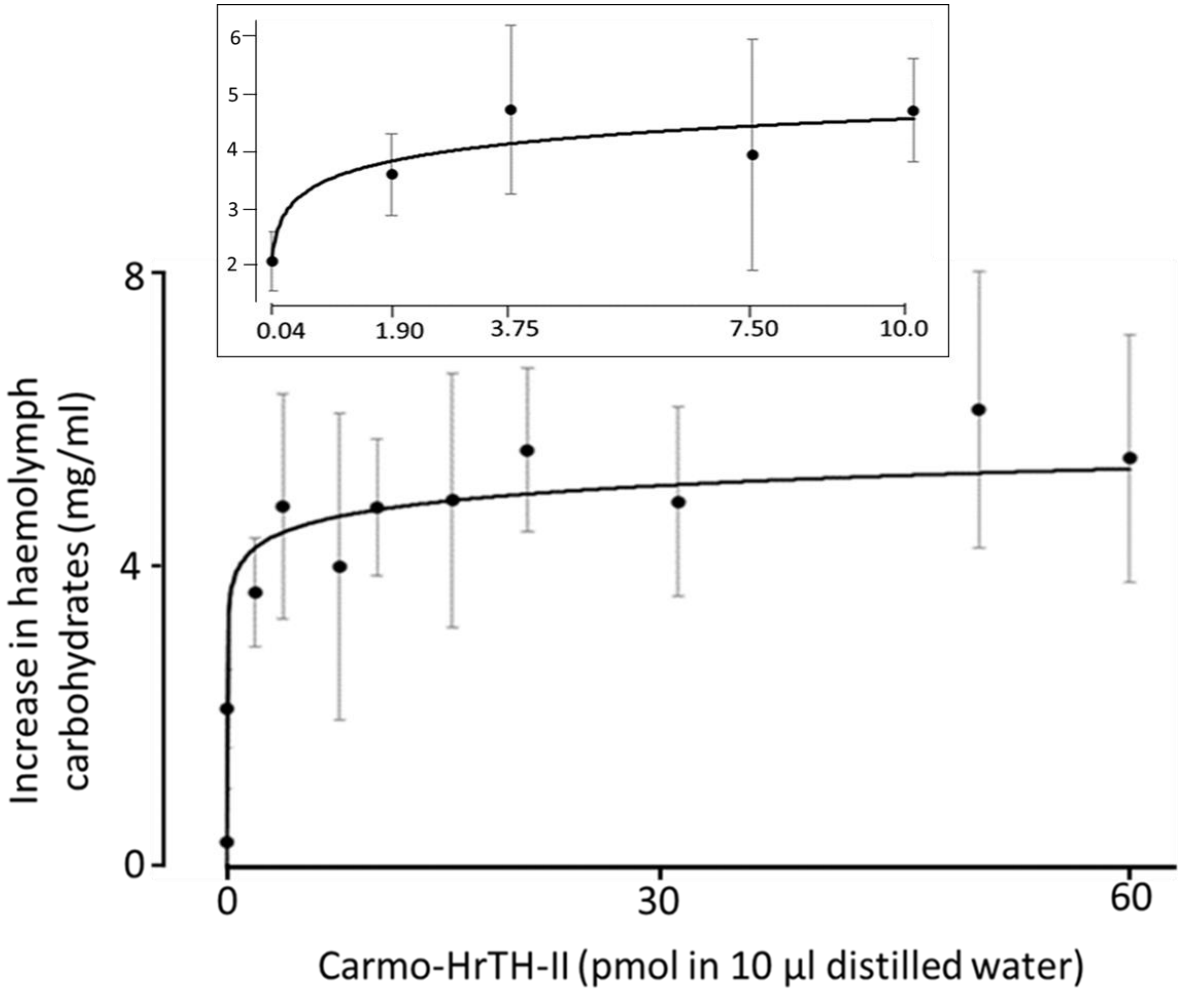

Figure 3.4. Dose-response relationship for Carmo-HrTH-II and the increase in haemolymph carbohydrates in ligated 6th instar nymphs of *C. morosus* (17 to 18 days old). Each data point represents the mean ± SD in mg/ml. At least 8 and no more than 12 insects were used for each dose. The inset is the magnification of the first 5 doses of the main graph.

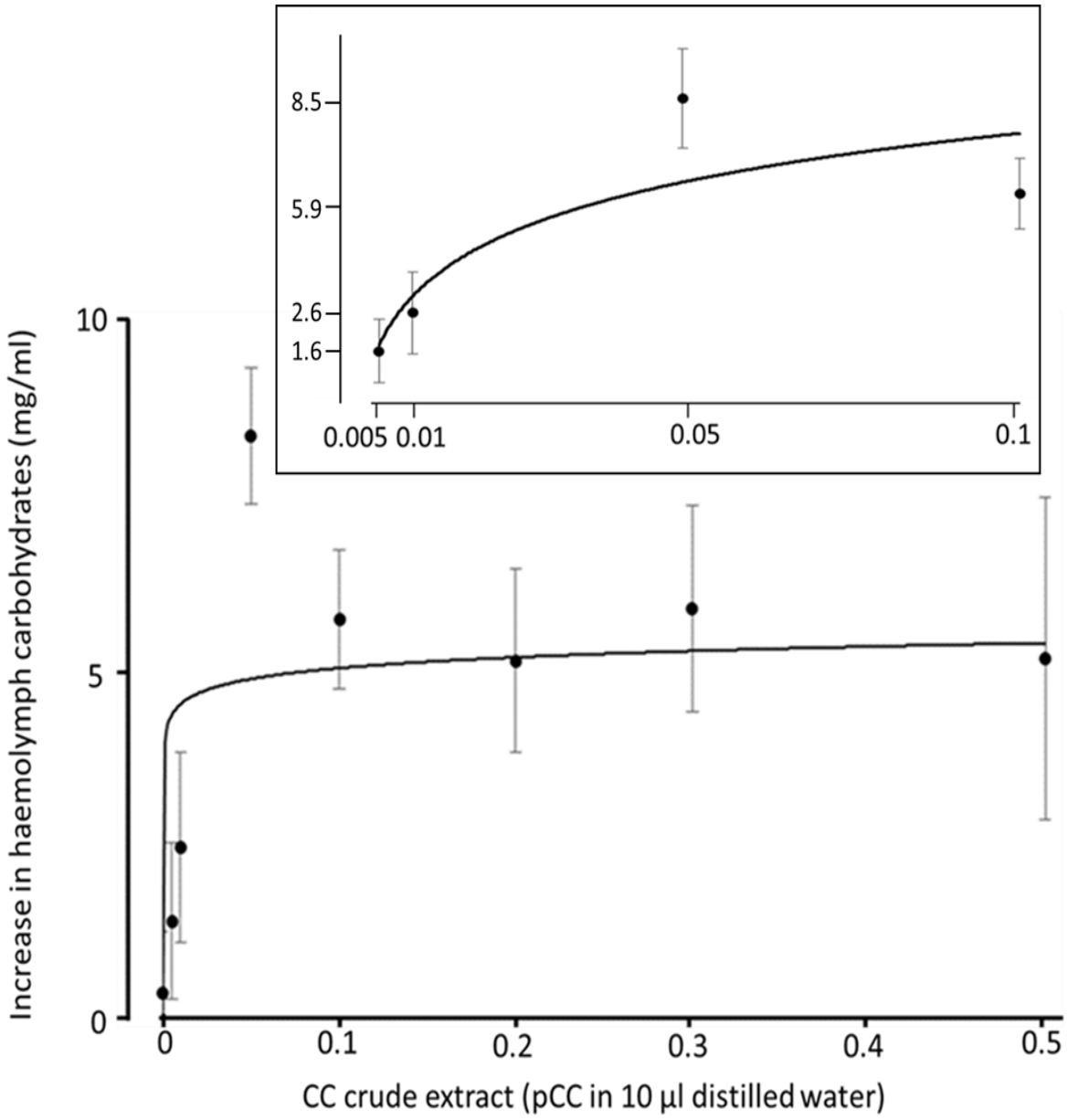

Figure 3.5. The effects of the increasing doses for the native CC extracts on carbohydrates mobilisation in ligated 6th instar nymphs of *C. morosus* (17 to 18 days old). Each data point represents the mean ± SD in mg/ml. At least 7 and no more than 11 insects were used for each dose. The small graph in the column is the magnification of the first four doses of the main graph.

3.3.2. Hypertrehalosaemic response of synthetic analogues of Carmo-HrTH-II in ligated 6th instar nymphs of C. morosus (17 to 18 days old)

3.3.2.1. N- and C-terminally modified analogues

The changes in the concentration of haemolymph carbohydrates in response to injections of the terminally modified analogues are shown in Fig. 3.6. A dose of 20 pmol of each of these peptides was injected. Distilled water and 20 pmol of Carmo-HrTH-II served as controls.

All the termini-modified analogues increased the haemolymph carbohydrates ($p < 0.05$) (Fig. 3.6). These increases, however, did not differ significantly from that caused by water ($p > 0.005$) but they differed from that of Carmo-HrTH-II ($F_{4, 56} = 21.817$, $p < 0.0005$). The percentages of the increase in haemolymph carbohydrates of these analogues, relative to the maximum increase caused by 20 pmol Carmo-HrTH-II are shown in Table 3.3.

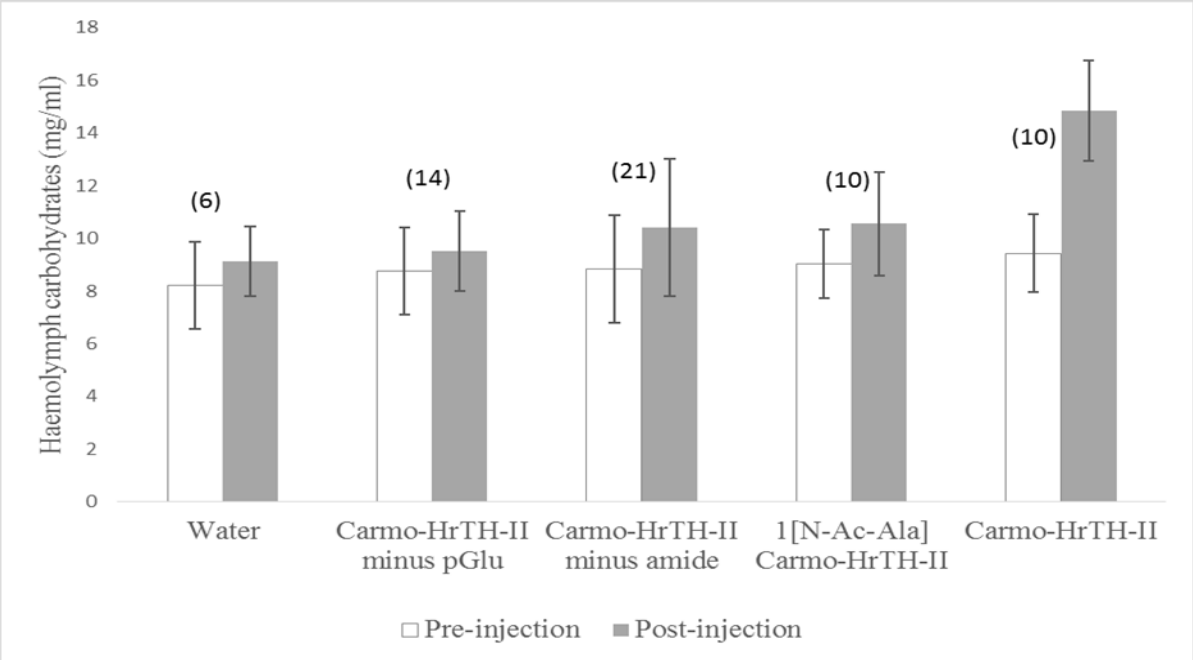

Figure 3.6. The hypertrehalosaemic effects of N- and C-terminally modified analogues of Carmo-HrTH-II assayed in ligated 6th instar nymphs of *C. morosus* (17 to 18 days old). A dose of 20 pmol of each of these analogues was injected. Each bar represents the mean ± SD, and the sample size is shown in parentheses. Student's paired t-test revealed that there was a significant difference ($p < 0.05$) between the pre- and post-injection values for each treatment. One-way ANOVA revealed that only Carmo-HrTH-II differs significantly from the rest of the other groups ($F_{4, 56} = 21.817$, $p < 0.0005$).

3.3.2.2. Naturally-occurring AKH octapeptides

The changes in the concentration of haemolymph carbohydrates in response to the injections of synthetic AKH octapeptides are shown in Fig. 3.7. A dose of 20 pmol of each of the octapeptides was injected. Distilled water and 0.2 pairs of native CC extract served as controls. Peram-CAH-II increased the haemolymph carbohydrates significantly ($p < 0.05$) while Aedae-AKH and Pyrap-AKH did not (Fig. 3.7.). There was a significant difference between the tested groups, controls included ($F_{4, 61} = 17.460$, $p < 0.0005$). The post hoc Scheffe's test revealed that the rest of the groups were different to 0.2 pCC ($p < 0.05$), and there was no significant difference between the octapeptides and water ($p > 0.05$). The percentage increase of the concentration of carbohydrates in haemolymph caused by these octapeptides, relative to that caused by 20 pmol Carmo-HrTH-II are shown in Table 3.3.

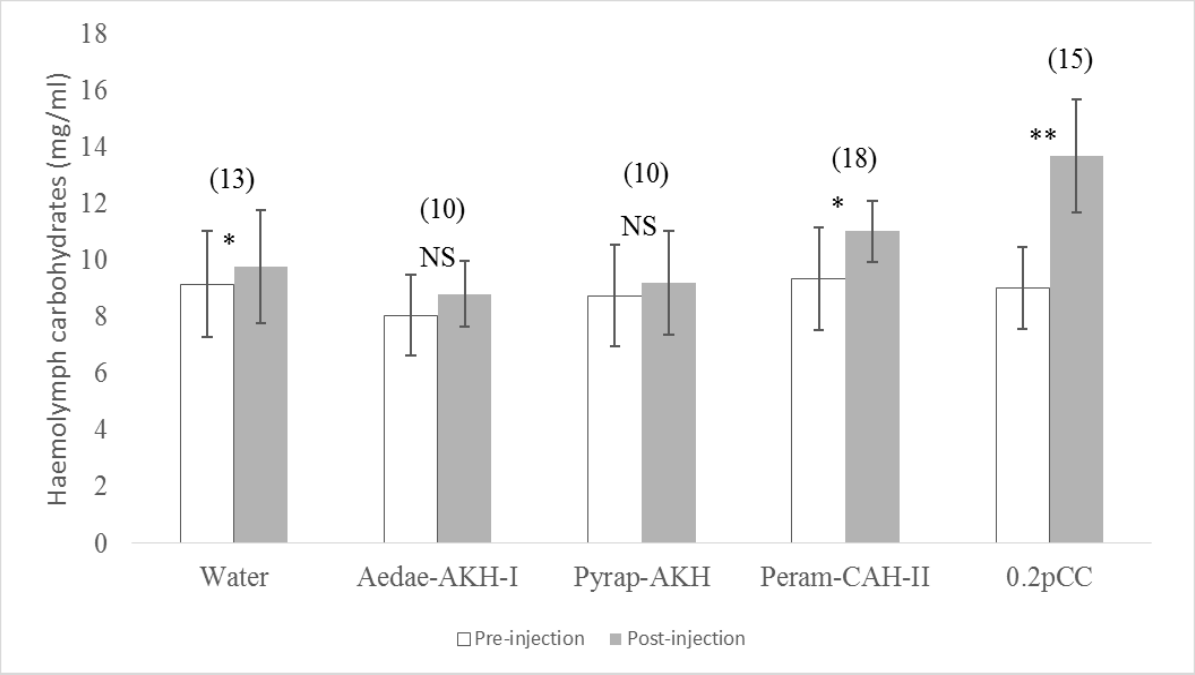

Figure 3.7. Biological activity of naturally-occurring AKH **octapeptides** assayed in ligated 6th instar nymphs of *C. morosus* (17 to 18 days old). Insects were injected with 20 pmol of the peptide. Distilled water and 0.2 pCC of native extract served as controls. Each bar represents the mean ± SD, and the sample size is shown in brackets. NS = no significant difference between pre- and post-injection values. A single asterisk represents a significant difference ($p < 0.05$) and double asterisk represents a highly significant difference ($p < 0.00005$).

3.3.2.3. Naturally-occurring AKH decapeptides and systematically altered analogues

All the analogues (decapeptides and systematically altered) were tested with the same dose (20 pmol) as that used for the endogenous peptides (Carmo-HrTH-I and -II). Distilled water and 0.02% acetonitrile were used as negative control. These controls were used because the AKH decapeptides were contained in water and systematically altered analogues were contained in acetonitrile when injected into test animals.

The change in concentration of carbohydrates in the haemolymph before and after the injection of analogues and their respective statistical significances are depicted in Figs. 3.8 and 3.9. The percentage increase of the carbohydrates in the haemolymph caused by the analogues, relative to the maximum increase caused by 20 pmol Carmo-HrTH-II, are presented in Table 3.3. The one-way ANOVA followed by post hoc Scheffe's test revealed that there was a significant difference among the natural occurring decapeptides and systematically altered analogues ($F_{5, 61}$ = 35.609, $p < 0.0005$; $F_{12, 177}$ = 49.811, $p < 0.0005$). Water and acetonitrile elicited less than 10 % of the hypertrehalosaemic activity. Only Phyle-CC and [Ala^2]-Carmo-HrTH-II increased the haemolymph carbohydrates as high as Carmo-HrTH-II ($p > 0.05$). Rommi-CC, [Ala^4]-Carmo-HrTH-II and [Ala^{10}]-Carmo-HrTH-II elicited about 46–56% of the maximum hypertrehalosaemic effects. Effects for this group differed significantly from that of water and acetonitrile ($p < 0.005$). The rest of the analogues were nearly unable to increase the haemolymph carbohydrates, only reached about 30% of the maximum activity produced by Carmo-HrTH-II. Effects did not differ significantly ($p > 0.05$) from that of water and acetonitrile.

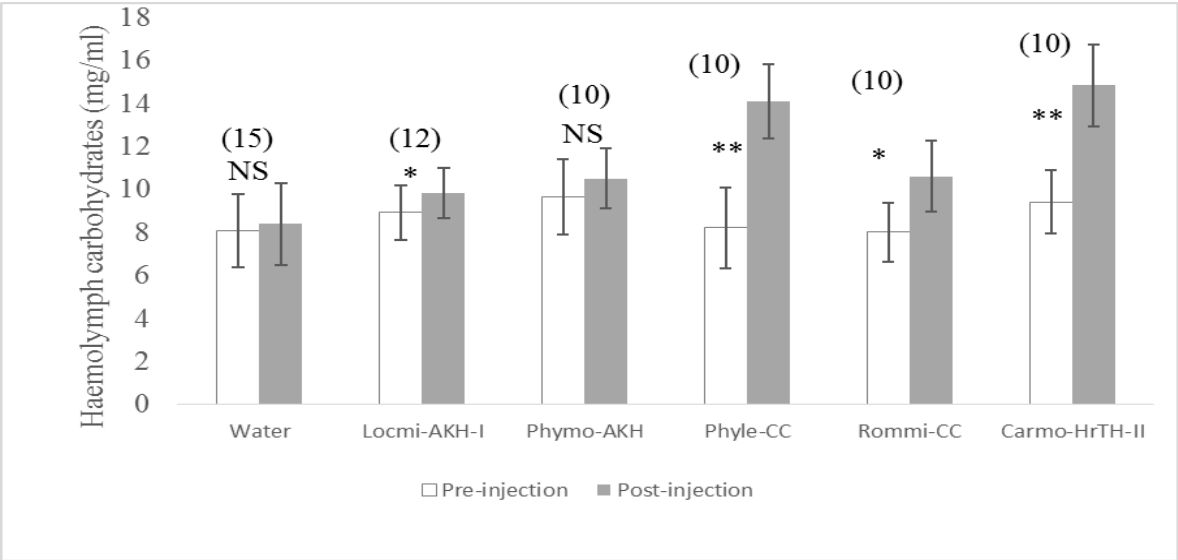

Figure 3.8. Biological activity of naturally-occurring AKH **decapeptides** assayed in ligated 6th instar nymphs of *C. morosus* (17 to 18 days old). Insects were injected with 20 pmol of the peptide. Each bar represents the mean ± SD, and the sample size is shown in brackets. NS = no significant difference between pre- and post-injection values. A single asterisk represents a significant difference ($p < 0.05$) and double asterisk represents a highly significant difference ($p < 0.00005$).

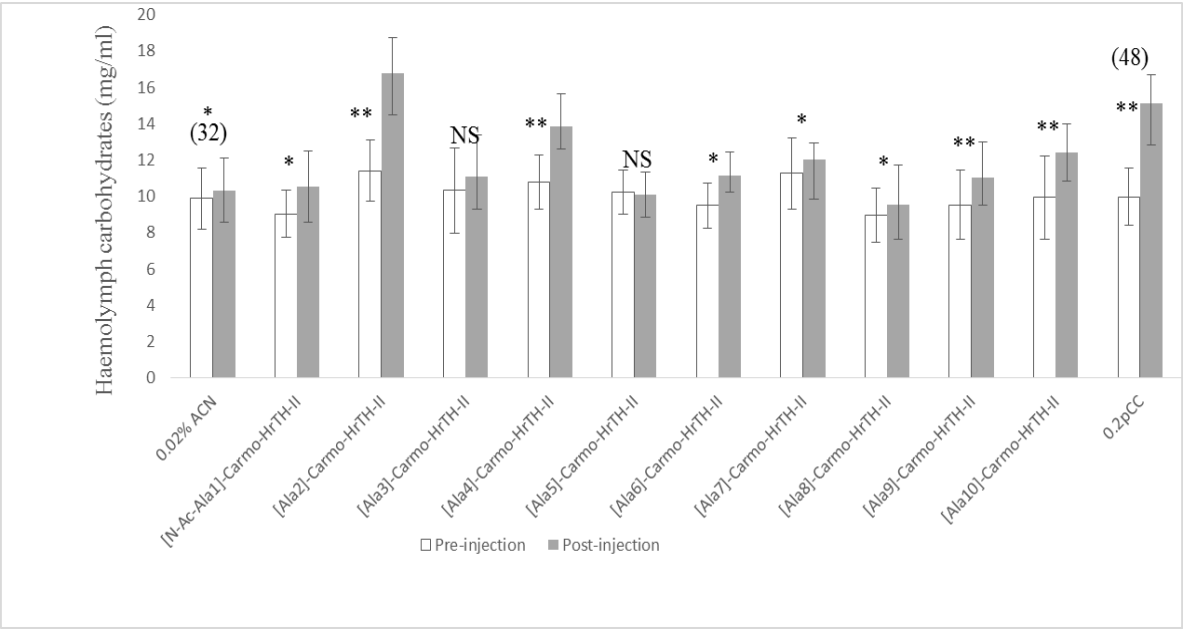

Figure 3.9. Biological activities of systematically altered analogues of Carmo-HrTH-II assayed in ligated 6th instar nymphs of *C. morosus* (17 to 18 days old). Insects were injected with 20 pmol of the peptide. Each bar represents the mean ± SD, and the sample size is 10 unless otherwise indicated in brackets. NS = no significant difference between pre- and post-injection values. A single asterisk represents a significant difference ($p < 0.05$) and double asterisk represents a highly significant difference ($p < 0.00005$).

Table 3.3. Peptides tested in *C. morosus* and their relative hypertrehalosaemic effects (synthetic Carmo-HrTH-II =100%).

Code-name of a peptide	Hypertrehalosaemic activity (%) relative to the maximal increase
Carmo-HrTH-II	100
Carmo-HrTH-I^{n}	93^{a}
Carmo-HrTH-II^{n}	88^{a}
Aedae-AKH	15
Peram-CAH-II	31
Pyrap-AKH	9
Locmi-AKH-I	17
Phyle-CC	109^{a}
Phymo-AKH	17
Rommi-CC	48
Carmo-HrTH-II minus amide	30
Carmo-HrTH-II minus pGlu	15
[N-Ac-Ala^{1}]- Carmo-HrTH-II	28
[Ala^{2}]- Carmo-HrTH-II	100^{a}
[Ala^{3}]- Carmo-HrTH-II	15
[Ala^{4}]-Carmo-HrTH-II	56
[Ala^{5}]- Carmo-HrTH-II	-2
[Ala^{6}]- Carmo-HrTH-II	31
[Ala^{7}]- Carmo-HrTH-II	13
[Ala^{8}]- Carmo-HrTH-II	11
[Ala^{9}]- Carmo-HrTH-II	28
[Ala^{10}]- Carmo-HrTH-II	46

^{a} indicates percentage activity did not differ significantly from that of Carmo-HrTH-II (100%).
^{n} indicates native (non-synthetic) peptides obtained from the CC of *C. morosus*. For primary sequences, see Table 1.2.

3.4. Semi-exposed heart bioassays

3.4.1. Neuropeptide action on heart beat rate

Two sets of heart experiments were carried out. One set consisted of semi-exposed heart assays done on insects where the heads were attached to the bodies (i.e., intact preparations). Another set was consisting of the similar heart assays done on insects that were either decapitated or had their heads intact but ligated on the neck with the thread (i.e., decapitated/ligated preparations).

In intact preparations, the heart beat rate ranged from 29 to 63 beats/min with an average of 44 ± 8 beats/min (*n*= 43). In decapitated/ligated preparations, the heart beat rate ranged from 28 to 53 with an average of 38 ± 5 beats/min (*n* = 70). Although these heart beat rates differed significantly from each other ($F_{1, 111} = 20.203$, $p < 0.0005$) their contraction pattern was similar. From the microscopic observations (not shown), the contraction pattern was a wave-like pulse associated with a few increases in diameter and asynchronous contractions. The direction of the flow of haemolymph was always anterograde. The preparations remained stable for several hours.

The first series of experiments involved the application of different doses of the native CC extracts (0.1 to 1 pCC) and Carmo-HrTH-II (5 to 80 pmol) on a few heart preparations of intact insects. There was no noticeable increase in heart beat rate for these preparations (results not shown, *n* = 3). There was, however, a noticeable change in the beating pattern after the application of the CC extract and Carmo-HrTH-II (dose ≥ 10 pmol). The pattern changed to a more pronounced wave-like pulse associated with several asynchronous contractions that would pause for several seconds. The lumen of the heart also widened in diameter. These observations were mostly seen within 6 minutes post-application (microscopic observation, not shown). Thereafter, several preparations were tested with

Carmo-HrTH-II again and a few of its analogues (20 to 40 pmol). There was still no increase in heart beat rate of the latter preparations ($p > 0.05$) (Fig. 3.10). There was, however, a noticeable change in the beating pattern post application of the Carmo-HrTH-II, Phyle-CC and Carmo-HrTH-II minus amide.

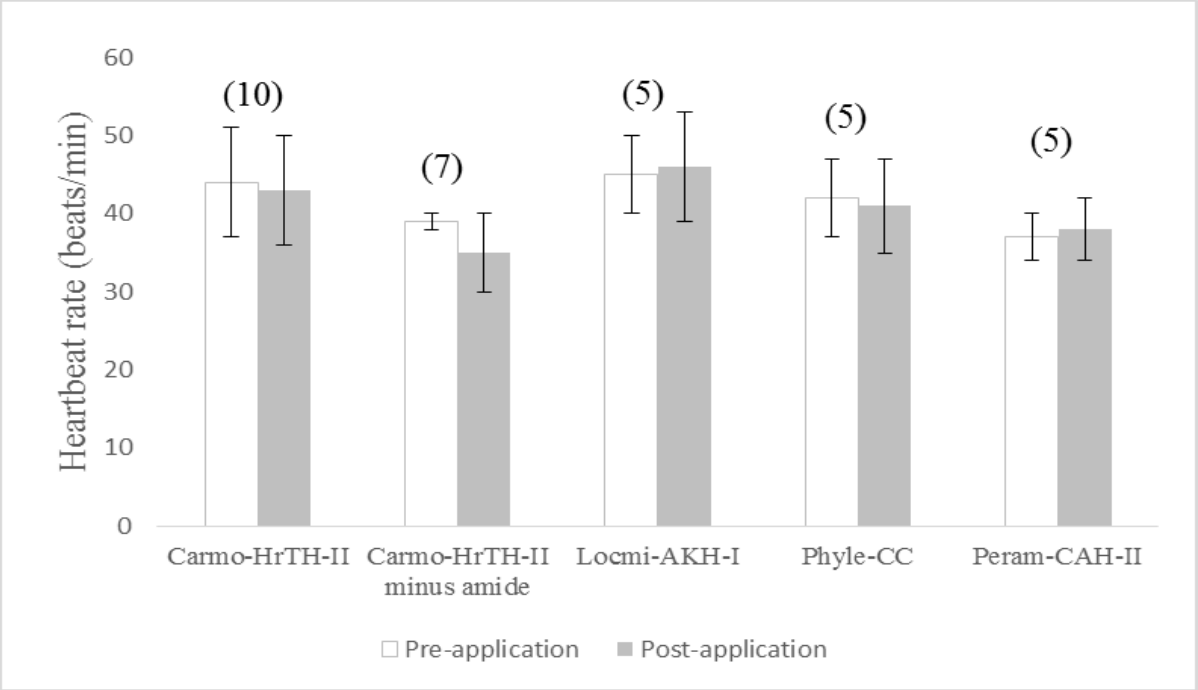

Figure 3.10. The effect of Carmo-HrTH-II and its analogues on the semi-exposed heart of intact adult *C. morosus* (1 to 2 months old). A dose of 20 pmol was applied to the heart for all the peptides except for Carmo-HrTH-II minus amide, which was 40 pmol. Each bar represents the mean ± SD, and the sample size is shown in brackets. There was no significant difference between the pre- and post-application values ($p > 0.05$).

The application of 150 µl saline containing Carmo-HrTH-II (10 to 110 pmol) on the heart preparation of insects that were either decapitated or neck-ligated increased the heart beat rate significantly ($p < 0.005$) (Fig. 3.11) and the pattern remained the same (pre- and post-application). Experiments studying the effects of various analogues of Carmo-HrTH-II, proctolin, CCAP and octopamine on heart beat rate were performed on semi-exposed hearts of neck-ligated insects.

A dose of 20 pmol was used to test all the naturally-occurring AKH peptides, termini modified and systematically altered analogues of Carmo-HrTH-II. As shown in Table 3.4, Carmo-HrTH-I and -II and termini modified analogues (Carmo-HrTH-II minus amide/pGlu) increased the heart beat rate significantly, and there was also a significant difference among these groups ($F_{4, 35} = 27.260$, $p < 0.0005$). The post hoc test revealed that there was no significant difference between Carmo-HrTH-I and -II ($p > 0.05$). There was a significant difference between Carmo-HrTH-II and these termini modified analogues.

There was a significant difference between the groups tested with different naturally-occurring peptides (Table 3.4) ($F_{7, 44} = 30.450$, $p < 0.0005$). The increase in heart beat rate caused by Rommi-CC and Phyle-CC (18 and 8 beats/min, respectively) did not differ significantly from that caused by Carmo-HrTH-II ($p > 0.05$).

As shown in Table 3.4, all the systematically altered analogues of Carmo-HrTH-II did not increase the heart beat rate significantly except for [Ala^3]-Carmo-HrTH-II. The one-way ANOVA followed by the post hoc Scheffe's test revealed that the [Ala^3]-Carmo-HrTH-II did not differ significantly from the rest of the analogues nor did it differ from saline ($p > 0.05$). All the systematically altered analogues and saline differed from Carmo-HrTH-II ($F_{11, 58} = 18.129$, $p < 0.0005$).

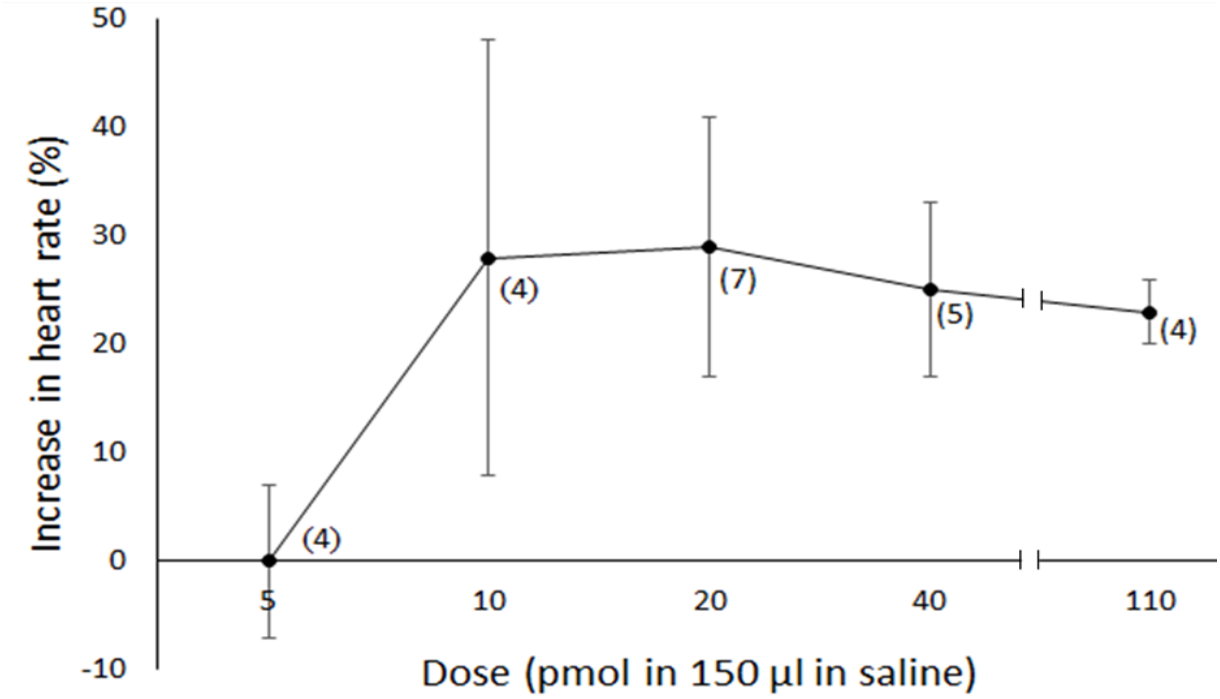

Figure 3.11. Dose-response curve for Carmo-HrTH-II on the semi-exposed heart of decapitated/ligated adult *C. morosus* (1 to 2 months old). Each bar represents the mean ± SD, and the sample size is shown in brackets. Note that the peptides and the control saline were applied in a volume of 150 µl.

Table 3.4. The increase in heart beat rate of semi-exposed heart preparations of *C. morosus* following the application of various synthetic AKH peptides and systematically altered analogues of Carmo-HrTH-II. A dose of 20 pmol in 150 µ saline for each analogue was applied. The percentage increase relative to that of 20 pmol Carmo-HrTH-II is shown.

Peptide name	*n*	Heart rate (beats/min)				% cardio-stimulatory activity relative to maximal increase
		Pre-A	Post-A	△	P	
Carmo-HrTH-II	8	41 ± 4	53 ± 5	12 ± 5	0.0002	100
Carmo-HrTH-I	4	41 ± 2	54 ± 2	13 ± 2	0.002	108[a]
Carmo-HrTH-II minus amide	7	40 ± 5	47 ± 3	7 ± 3	0.0008	58
Carmo-HrTH-II minus pGlu	9	39 ± 6	46 ± 5	7 ± 2	0.000001	58
Saline	12	40 ± 4	41 ± 1	1 ± 2	NS	8

Peptide name	*n*	Heart rate (beats/min)				% cardio-stimulatory activity relative to maximal increase
		Pre-A	Post-A	△	P	
Carmo-HrTH-II	8	41 ± 4	53 ± 5	12 ± 5	0.0002	100
Aedae-AKH	5	42 ± 6	43 ± 6	1 ± 1	0.03	8
Locmi-AKH-I	4	40 ± 4	43 ± 4	3 ± 1	0.002	25
Phyle-CC	5	38 ± 7	46 ± 6	8 ± 2	0.0005	67[a]
Phymo-AKH	6	37 ± 3	38 ± 4	1 ± 1	0.04	8
Pyrap-AKH	6	39 ± 5	41 ± 4	2 ± 2	0.02	16
Rommi-CC	6	39 ± 3	57 ± 4	18 ± 6	0.0007	150[a]
Saline	12	40 ± 4	41 ± 1	1 ± 2	NS	8

Peptide name	*n*	Heart rate (beats/min)				% cardio-stimulatory activity relative to maximal increase
		Pre-A	Post-A	△	P	
Carmo-HrTH-II	8	41 ± 4	53 ± 5	12 ± 5	0.0002	100
[N-Ac-Ala^{1}]	8	39±6	39±6	0±1	NS	0
[Ala^{2}]	5	42±7	42±5	0±2	NS	0
[Ala^{3}]	4	38±6	40±6	2±1	0.035	16
[Ala^{4}]	5	40±4	41±4	1±1	NS	8
[Ala^{5}]	5	39±7	40±7	1±2	NS	8
[Ala^{6}]	5	43±5	43±5	0±1	NS	0
[Ala^{7}]	5	39±4	39±4	0±1	NS	0
[Ala^{8}]	5	42±5	42±4	0±2	NS	0
[Ala^{9}]	4	39±2	38±2	-1±1	NS	-8
[Ala^{10}]	4	41±5	41±5	0±1	NS	0
Saline	12	40 ± 4	41 ± 1	1 ± 2	NS	8

Data presented as mean ± SD for pre-application (Pre-A), post-application (Post-A) and the difference (△) between pre- and post-application values.
No significance is represented by NS.
Superscript "a" indicates that percentage increase in heart did not differ significantly from that of Carmo-HrTH-II (100%).

The insect cardio-stimulatory peptides, proctolin and CCAP, were used to determine the maximum increase in heart beat rate of *C. morosus*. Hence, different doses of these peptides contained in 150 µl saline, were applied on the semi-exposed hearts of ligated adult *C. morosus*. Normal saline (150 µl) severed as a control. As depicted in Fig. 3.12 and Table 3.5, both proctolin and CCAP increased the heart beat rate in a dose-dependent manner. Doses of 0.04 pmol of proctolin and 0.015 pmol of CCAP, were sufficient to increase the heart beat rate by about 20%. Doses of 0.5 pmol of proctolin and 150 pmol of CCAP caused the heart to beat very fast for about 2 minutes and then led to tetanus of the heart muscles (no visible contractions). Any dose higher than this resulted in immediate stopping of heart contractions. When doses of 0.3 pmol of proctolin and 0. 75 pmol of CCAP were tested on the semi-exposed heart of intact *C. morosus* (n=5 for both peptides), heart beat rate increased by about 50%.

The application of the neurotransmitter octopamine had an inhibitory effect on the contraction the semi-exposed heart of *C. morosus* (Fig.3.13). For each concentration, eight counts in beats/ min were done subsequently within 14 minutes post application of the test solution. A concentration of 10^{-2} M octopamine increased the heart beat rate by about 80% immediately after application, but this effect only lasted for about 1.5 min. The heart beat rate then decreased by 60% and then remained beating slowly until the test solution was washed off at about 15 min. With a dose of 10^{-3} M octopamine, the heart rate remained more or less the same for about 1.5 min, then decreased by more than 50%. With 5×10^{-4} M and 10^{-4} M octopamine, the heart beat rate decreased by about 30% immediately after application.

Table 3.5. The increase in heart beat rate of semi-exposed heart preparations of *C. morosus* following the application of different doses of CCAP.

Dose (pmol in 150 µl saline)	Heart rate (beats/min)					Increase in heart rate (%)
	n	Pre-A	Post-A	△	P	
Saline	5	39 ± 5	40 ± 5	1 ± 3	NS	3
0.015	4	42 ± 2	52 ± 4	10 ± 3	0.004	24
0.75	5	38 ± 3	56 ± 4	18 ± 1	0.00003	47
1.5	4	43 ± 2	62 ± 5	19 ± 3	0.001	44
150	4	44 ± 4	65 ± 3	21 ± 3	0.0009	48

Data presented as mean ± SD for pre-application (Pre-A), post-application (Post-A) and the difference (△) between pre- and post-application values. No significance is represented by NS.

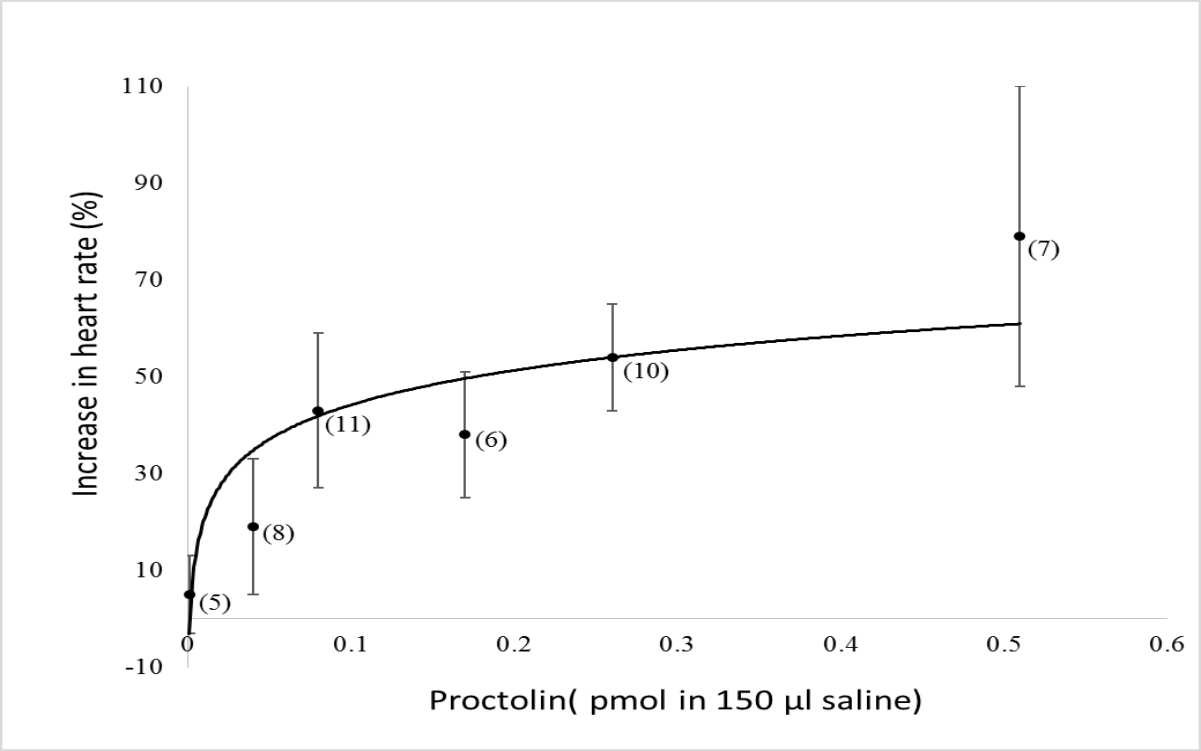

Figure 3.12. The effects of proctolin on the semi-exposed heart beat rate of ligated adult *C. morosus* (1 to 2 months old). Each data point represents the mean ± SD, and the sample size is shown in brackets. A mean heart beat rate (beats/min) was obtained over a period of 4 min after application of proctolin in all insects, except for the 0.5 pmol group, where the heart stopped beating after 2 min. In the latter group, therefore, a mean heart beat rate was calculated over a 1 min period after application of proctolin. Note that the peptides and the control saline were applied in a volume of 150 µl.

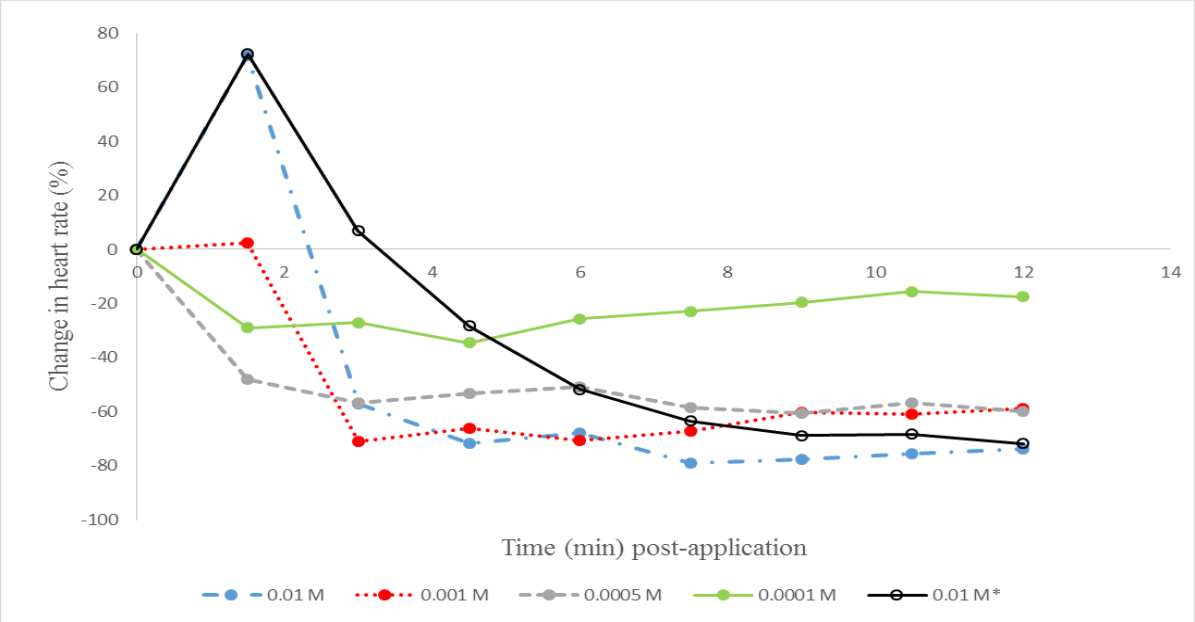

Figure 3.13. The effects of different concentrations of octopamine on the semi-exposed heart of adult *C. morosus* (1 to 2 months old). Each line represents the concentration of octopamine applied to the heart. The concentrations indicated in blue (0.01M), red (0.001M), grey (0.0005M) and green (0.0001M) were applied on preparation of ligated insects (*n* = 4). The concentration shown in black was applied on preparation of intact insects (n = 5).

4. Chapter four: Discussion

The peptides of the AKH/RPCH family are mainly known for their involvement in the regulation of energy metabolism, specifically the mobilising of the stored fuel metabolites (lipid, carbohydrates or proline) and also for their cardio-stimulatory effects (Gäde, 1997a; Gäde & Marco, 2013). The stimulation of the heart by AKH peptides is believed to be the mechanism for assisting the faster distribution of fuel metabolites (Gäde & Marco, 2013).

The present study aimed to investigate the importance of structural features of the native hypertrehalosaemic hormones (HrTHs) necessary to interact with the receptor of the fat body and dorsal vessel cells of *Carausius morosus* to trigger a response eventually leading to the release of carbohydrates in the haemolymph and an increase in heart beat rate. Two biological assays, carbohydrate-mobilisation and semi-exposed heart, were used to assess the biological activities of various synthetic preparations of naturally-occurring AKH peptides and systematically altered analogues in comparison to the endogenous HrTH-II.

4.1. Hypertrehalosaemic response in *C. morosus*

4.1.1. Hypertrehalosaemic effects of native hormones

The investigation of hypertrehalosaemic activity in the stick insect *C. morosus* started more than 35 years ago. Initially, studies were able to show that the corpora cardiaca (CC) of *C. morosus* contain adipokinetic/hypertrehalosaemic hormone(s) capable of mobilising carbohydrates in the cockroach *Periplaneta americana* and lipids in the locust, *Locusta migratoria* (Gäde, 1979, 1980). However, substrate mobilisation could not be demonstrated in the stick insects themselves during that time (Gäde, 1979, 1980). The *C. morosus* CC were also shown to be capable of elevating the cyclic AMP in *L. migratoria* (Gäde, 1979). In 1982, it was demonstrated, for the first time, the ability of the CC to mobilise the carbohydrates in *C. morosus* but only when the insects were ligated on the neck and not in the non-ligated

ones (Gäde & Lohr, 1982). These authors also demonstrated that two peaks, obtained from column chromatography of the *C. morosus* CC, were responsible for this mobilisation. The structures of these peaks were later determined and denoted as *C. morosus* hypertrehalosaemic hormones I and II, Carmo-HrTH-I and -II (Gäde, 1985; Gäde *et al*., 1992; Munte *et al*., 2008), which belong to the AKH/RPCH family. Gäde & Lohr (1982) characterised the hypertrehalosaemic actions of Carmo-HrTH-I and -II in *C. morosus*. The authors reported that stick insects (ligated) show maximum hypertrehalosaemic response when nymphs were about to moult into adults. This was the developmental stage when initial concentration of carbohydrates in the haemolymph were high (Lohr & Gäde, 1983). The present study confirmed these findings and worked with that same developmental stage to attain the maximum hypertrehalosaemic response. It is believed that ligation itself have no impact on the concentration of carbohydrates but limits the flow of haemolymph around the body (Gäde & Lohr, 1982). The inability to demonstrate hypertrehalosaemic activity in non-ligated stick insects suggests that there is an unknown antagonistic action initiated in the head of this insect to inhibit the mobilisation of carbohydrates. The inability of the native CC to mobilise carbohydrates nor lipids was also observed in another stick insect species *Cuniculina impigra* (Gäde, 1980). It is not shown yet whether the CC of *C. impigra* have hypertrehalosaemic effects in ligated *C. impigra* itself. However, it is known that the CC of *C. impigra* contain hypertrehalosaemic hormone (s) because they were capable of mobilising carbohydrates in ligated *C. morosus* (Gäde & Lohr, 1982). Malik *et al* (2012), demonstrated hypertrehalosaemic activity in ligated stick insect *Baculum extradentatum*. It is still, however, not known whether the latter stick insect also does not show hypertrehalosaemic activity when not ligated. Nevertheless, this ligation case seemed to occur only in Phasmatodea insects, it is not yet reported in other insect orders. Stick

insects have less fuel metabolites and are less active as compared to other insects such as cockroaches and locusts as (Lohr & Gäde, 1983). This may suggests that the main usage of stored carbohydrates in stick insects could be during moulting, for the synthesis of the new chitin (Lohr & Gäde, 1983) unlike in active insects where the carbohydrates (or lipids) are mostly required for energy. Thus, stick insects might have strategies to then prevent the mobilisation of carbohydrates when not going through activities that required carbohydrates regardless of the presence of the hormones responsible. The maximum response to the hypertrehalosaemic observed in nymphs about to moult in the present study, as well as that of Lohr & Gäde (1983) could be related to the moulting process.

The present study showed that both Carmo-HrTH-I and -II have the same hypertrehalosaemic effect in *C. morosus*. These findings agree with those of Gäde & Lohr (1982). Carmo-HrTH-I and -II are both decapeptides and the only difference between the two is that Carmo-HrTH-I contains a mannose residue on its tryptophan. Hence, Carmo-HrTH-II was used as the lead peptide for the assessment of structural features. The assumption was made that there is only one HrTH receptor that is activated by both Carmo-HrTH-I and -II in *C. morosus*. Even though there are no reports on the identification of HrTH receptor(s) or any other G-protein coupled receptors (GPCRs) in *C. morosus*, these assumptions were based on evidence from other insects. Studies that used gene cloning techniques, for example, reported individual insects to contain only one AKH/HrTH receptor, even those that have more than one AKH/HrTH. For example, *P. americana*, (have two HrTHs) but only one HrTH receptor was identified, which is activated by both the native HrTH ligands (Hansen *et al*., 2006). In *Bombyx mori*, which also contains two AKHs only one AKH receptor was identified (Staubli *et al.*, 2002; Zhu *et al.*, 2009). The interaction of Carmo-HrTH-I and -II with the respective receptor will be discussed later.

4.1.2. Importance of structural features of Carmo-HrTH-II

4.1.2.1. N- and C-terminally modified analogue

One of the common characteristics of peptides from the AKH/RPCH family are blocked termini: pGlu residue at the N-terminus and an amide at the C-terminus. These features are a result of post-translational modifications of the peptide (Gäde, 2009), which provide protection against exopeptidases (Gäde & Hayes, 1995). The current study assessed the importance of blocking residues by testing three analogues: Carmo-HrTH-II without an amide or **(**pGlu) and analogue where the pGlu was replaced with an N-acetyl-alanine residue (N-Ac-Ala). All these analogues showed no hypertrehalosaemic activity, even when the N-terminal end was blocked by an acetyl-Ala. Thus, the N-terminal pGlu and C-terminal amide are important structural features essential for biological activities in stick insects. Previous work on other insects had also reported reduced or no activity when one of the terminal-blocking residues was removed from the native AKHs or when the pGlu was substituted with other blocked amino acids (Stone *et al.*, 1978; Gäde, 1990; Ziegler *et al.*, 1991,1998; Gäde & Hayes, 1995; Lee *et al.*, 1997; Marco & Gäde, 2015). Some studies that used *in vitro* receptor binding assays to investigate the accessibility of the ligand to the expressed AKH receptors of flies reported a decline in binding when the N-terminal acetylated-Ala analogue of the native AKH was tested (Caers *et al.*, 2012, 2016).

Marco & Gäde (2015) mentioned that the negative charge introduced at the C-terminus after deamination does not have a major impact on the biological activities *in vivo.* Hence, it is most likely that the loss of activity is because the deaminated analogue was not protected from the exopeptidases, which results in the peptide being digested in the haemolymph before reaching its respective receptor in *in vivo* bioassays (Marco & Gäde, 2015). This implies that the 40-70% biological activities caused by the non-amidated native peptides for

flies (*D. melanogaster* and *Glossina morsitans morsitans*) in *in vitro* assays with the expressed AKH receptors (Caers *et al*., 2012; Caers *et al*., 2016) could be because the analogues were not subjected to carboxypeptidases (Marco & Gäde, 2015). NMR studies done on various AKH peptides also predicted the presence of a β- turn at the position of 5 to 8 (Hayes & Keeley, 1990; Gäde, 1992; Zubrzycki & Gäde, 1994; Cusinato *et al.*, 1998). Hayes & Keeley (1990) predicted that the pGlu and the amide residues on the termini of the decapeptide HrTH of the cockroach *Blaberus discoidalis* (Bladi-HrTH) interact with each other to stabilise an assumed turn-induced folded conformation. If this is applied to all other AKH/HrTH peptides including Carmo-HrTH-II (Fig. 4.1), then removing one of these residues should not only expose the peptide to exopeptidases, but should also weaken the stability of the peptide which in turn will affect the binding of the peptide to its receptor.

4.1.2.2. The importance of aromatic amino acids, Phe and Trp

Another characteristic feature of the AKH/RPCH peptides is the presence of the aromatic amino acids at position 4 (Phe or Tyr) and 8 (Trp) (Gäde, 2009). In case of the stick insect *C. morosus*, Carmo-HrTH-I has a Trp residue at position 8, but it is modified. This Trp residue contains a hexose attached to its carbon-2 (Gäde *et al.*, 1992; Munte *et al.*, 2008). In the stick insect *Baculum extradentatum*, an AKH-like peptide with unknown function was isolated together with Carmo-HrTH-II (Malik *et al.*, 2012). This AKH-like peptide has all the structural features of AKH/RPCH peptides except the Trp^{8} is modified to a kynurenine (Malik *et al.*, 2012).

According to the present study and that of Gäde & Lohr (1982), both Carmo-HrTH-I and -II have the same hypertrehalosaemic activity in *C. morosus*. One assumes that these results may be an indication that the mannose residue on Trp^8 of Carmo-HrTH-I plays no role in receptor binding and activation, or that the mannose residue is dismantled in the haemolymph before the peptide bind to the receptor.

The relevance of the aromatic side chain of Trp^8 and Phe^4 of Carmo-HrTH-II was examined in the present study using analogues with a single replacement of these residues by Ala (Ala^4- and Ala^8-Carmo-HrTH-II, respectively). The removal of the aromatic side chain of Phe resulted in 44% loss of hypertrehalosaemic activities while that of Trp showed no activity. These may be an indication that in *C. morosus* the indole ring at position 8 is favoured more than the phenyl ring at position 4. Velentza *et al*., (2000) reported that in *L. migratoria* the single substitution of Phe^4 with Trp of Locmi-AKH-I is tolerated while the replacement of Trp^8 with Phe is not. These results from the two substitutions might entail that the Trp is a more important at structural feature than Phe. Previous studies, using *in vivo* or *in vitro* assays to assess receptor-peptide interaction, reported trace or no biological activities when either one of these aromatic amino acids were removed, which signifies that these structural features are crucial for receptor-binding in insects (Stone *et al.*, 1978; Gäde & Hayes, 1995; Ziegler *et al.*, 1998; Caers *et al.*, 2012, 2016; Marco & Gäde, 2015).

There have been no NMR studies and no modelling done on Carmo-HrTH-II. However, based on the predictions done on other AKHs such as Peram-CAH-I (Gäde, 1992), Bladi-HrTH (Hayes & Keeley, 1990) and Emppe-AKH (Zubrzycki & Gäde, 1994), it is assumed that Carmo-HrTH-II contains a β-turn at position 5 to 8. If this is true, then the two aromatic amino acids Phe^4 and Trp^8 are predicted to interact with each other (Fig.4.1). Therefore, removing one of

the aromatic side chains will disrupt this interaction, which will have an impact on the ability of the peptide to bind to its receptor successfully.

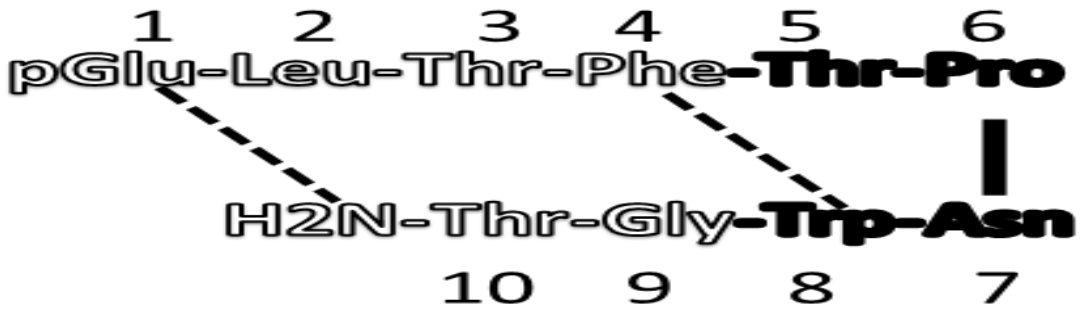

Figure 4.1. Predicted schematic diagram of the sequence of **Carmo-HrTH-II** in a folded conformation. Residues 5 to 8 in bold indicate the potential β-turn. The dashed lines indicate the possible interactions present in the conformation. The image is drawn based on Hayes & Keeley (1990), for the cockroach *Blaberus discoidalis* HrTH.

4.1.2.3. The importance of octapeptides

The chain lengths of the members of the AKH/RPCH family vary from eight to ten amino acids. The HrTHs of *C. morosus* are ten amino acids long. In this study, three naturally-occurring AKH octapeptides were assayed for hypertrehalosaemic in *C. morosus*. These assays revealed that at least 70% of activities were lost for all the octapeptides in comparison to the native peptide. This was even true of the cockroach peptide, Peram-CAH-II, which is identical at the N-terminal with Carmo-HrTH-II but lacks the two additional amino acids present at the C-terminal end. The CC extract of *P. americana* (containing Peram-CAH-II) showed the same hypertrehalosaemic activity in *C. morosus* (Gäde & Lohr, 1982) as that of Peram-CAH-II in the present study. The decline in these biological activities could mean that the HrTH receptor(s) of *C. morosus* have a weak affinity for the octapeptides, suggesting that the two amino acids (Thr and Gly) at the C-terminal end of the native peptides are needed for interaction. Since these AKH peptides are predicted to

consist of a β-turn at position 5 to 8 (Hayes & Keeley, 1990; Gäde, 1992; Zubrzycki & Gäde, 1994; Cusinato *et al.*, 1998), there should be a difference in conformations of the octapeptides and decapeptides (Figs. 4.1 and 4.2), which might be crucial for peptide-receptor binding. The β-turn of the octapeptide is more shifted to the C-terminal in comparison to that of the decapeptide. This could cause the differences in the interaction between N- and C-terminal ends of these peptides. These differences, however, did not seem to have major impacts on the HrTH receptor of *P. americana*; Carmo-HrTH-II has been reported to activate this receptor (Gäde, 1990).

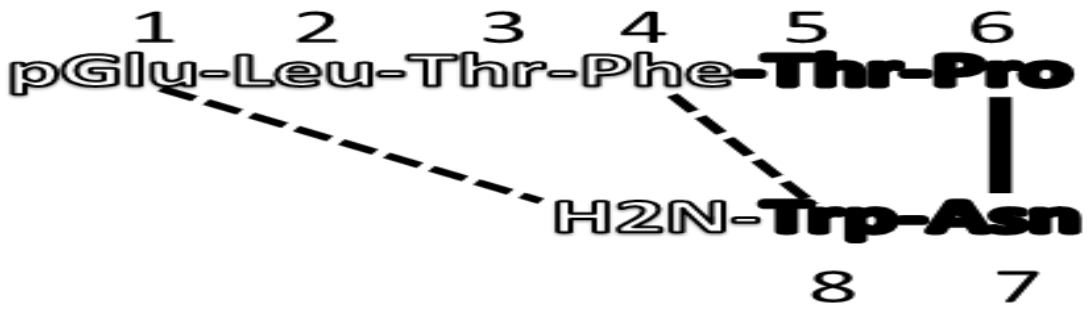

Figure 4.2. Predicted schematic diagram of the sequence of **Peram-CAH-II** in a folded conformation. Residues 5 to 8 in bold indicate the potential β-turn. The dashed lines indicate the possible interactions present in the conformation. The image is drawn based on Gäde (1992) as well as Hayes & Keeley (1990).

4.1.2.4. The importance of side chains and amino acid replacement

The results of the current study revealed that only Phyle-CC and Ala^{2}-Carmo-HrTH-II out of 14 decapeptides mobilised carbohydrates in *C. morosus* as much as the native peptides. Another two peptides Rommi-CC and Ala^{4}-Carmo-HrTH-II had about 50% hypertrehalosaemic activities. The remaining ten decapeptides showed poor or no hypertrehalosaemic activities. The data suggest that most amino acids and side chains of the native peptides are important for receptor recognition.

It is not surprising that Phyle-CC increased the concentration of carbohydrates in the haemolymph of C. morosus as much as Carmo-HrTH-II. Phyle-CC only differs from Carmo-HrTH-II at position 10 by having Ser instead of Thr. Previous work done on *P. americana* and *L. migratoria* has shown that Ser and Thr can replace each other without effecting the potency of the native peptide (Gäde, 1992, 1997c). The unchanged potency might be because both Thr and Ser are polar amino acids with hydroxylated side chains. In this study, the single Ala replacement of Thr at position 3, 5 or 10 of Carmo-HrTH-II, resulted in the complete loss of or decline in biological activity. This may be because Ala is non-polar and lacks a hydroxylated side chain. The replacement of Thr^{3} for Ala resulted in no activity in previous studies that used an *in vitro* receptor binding assay with expressed AKH receptors of dipteran insects. These replacements were done on native peptides (Drome-AKH and Glomo-AKH) that are similar to Carmo-HrTH-II at position 1 to 4 (Caers *et al.*, 2012, 2016).

The replacement of Thr with Ala at position 3 and 5 disrupted the alternating hydrophilic amino acids pattern (Thr^{3}, Thr^{5}, Asn^{7}) of Carmo-HrTH-II, which in turn might interrupt peptide conformation and thus effect the binding efficacy of the peptide. Replacing Thr with Ala at position 10 of Carmo-HrTH-II increased the peptide hydrophobicity (Trp^{8}, Gly^{9}), but apparently this change is not very crucial as compared to the latter replacement 50%

activity recorded in the hypertrehalosaemic assay. This might also mean that the hydroxyl group present on the terminal end (Thr^{10}) is less important for peptide-receptor interaction compared with that present on non-terminal residues (Thr^{3}, Thr^{5}). Poulos *et al.*, (1994) emphasised that in *L. migratoria*, the hydroxyl group of the Thr^{5} of Locmi-AKH-I is important for activity while that of Thr^{10} is not. These authors also added that the hydrogen of the hydroxyl group of Thr^{10} does not contribute to the essential hydrogen bond stabilising the active conformation.

The importance of the hydroxyl group was further supported by the replacement of Thr^{3} of Carmo-HrTH-II with Asn (Locmi-AKH-I), which showed no hypertrehalosaemic activities in *C. morosus* in the current study. These results are in agreement with those of Gäde & Lohr (1982). In the stick insect *B. extradentatum* that also have Carmo-HrTH-II, Locmi-AKH-I resulted in reduced hypertrehalosaemic activities in comparison to Carmo-HrTH-II (Malik *et al.*, 2012). The replacement of Thr^{3} with Asn^{3} that resulted in no biological activity (i.e., hyperlipemia) was also reported in *Hippotion eson*; Thr^{3} is conserved for all five native AKH peptides of this moth (Marco & Gäde, 2015).

Even though both amino acids (Asn and Thr) are hydrophilic, the loss in activity could be because Asn is lacking the hydroxyl group on its side chain, which is present on Thr (Marco & Gäde, 2015). The loss in activity could also be caused by the presence of the carboxamide on Asn. The results imply that even the removal of a simple side chain, such as a hydroxyl group, can be quite crucial for ligand-receptor interaction, as was seen earlier with the replacement of Thr with Ala. However, the addition of the hydroxyl group and the removal of the carboxamide did not have any impact on the receptors of *L. migratoria*. Carmo-HrTH-II was reported to be equally active as Locmi-AKH-I in this locust, and all the native AKH

peptides of this locust have Asn^3 (Gäde, 1990). This suggests that the AKH/HrTH receptors of the stick insects and locusts differ.

Additionally, *P. americana* contains one hypertrehalosaemic receptor (Hansen *et al.*, 2006) which is activated by both its native peptides, Peram-CAH-I and -II (Scarborough *et al.*, 1984; Gäde, 1990). The interchangeability of Asn and Thr is tolerated because Peram-CAH-I contains Asn in position 3 and Peram-CAH-II contains Thr in the same position. The *in vitro* receptor binding assay has shown that Peram-CAH-II activated the receptor slightly more than Peram-CAH-I (Hansen *et al.*, 2006), which suggests that the hydroxyl group gives the peptide a stronger affinity for its receptor.

The current study revealed that the substitution of Leu^2 in Carmo-HrTH-II with Ala has no impact on the HrTH receptor on the fat body of *C. morosus*. No loss in activity was recorded for this substitution in the *in vivo* carbohydrate bioassay. Previous work that used *in vitro* assays with the expressed AKH receptors of dipteran insects showed more than 50% decline in receptor activation when the Leu at position 2 of the native peptide was replaced with Ala (Caers *et al.*, 2012, 2016). Both Leu and Ala residues are non-polar. Hence, the alternating hydrophilic pattern of the peptide was not disrupted by the replacement. The only difference between these residues is that Leu has a bulkier alkyl side chain than Ala ($-CH_2CH(CH_3)_2$ vs. $-CH_3$), which makes it more non-polar. The different results obtained from the different assays suggest that the alkyl differences between Leu and Ala do not impact the peptide-receptor interaction *in vivo* but strongly impact the interaction *in vitro.*

It was not expected that the double replacement of Leu^2-Thr^3 with Val^2-Asn^3 (Rommi-CC) would elicit about half of the maximum hypertrehalosaemic activity This was different to the complete loss of activity caused by the single replacement of Thr^3 with Asn^3 (Locmi-AKH-

I) and the double replacement of Thr^3, Thr^{10} with Asn^3, Ser^{10} (Phymo-AKH). The hydrophilic alternating pattern was maintained by Rommi-CC. Having established already that the single replacement of Thr with Asn results no receptor activation, these data suggest that the long side chain of Val (as compared to Leu) could be the reason for restored activity. Gäde (1992) reported that Leu and Val at position 2 of Peram-CAH-II can be interchanged without effecting the affinity of the peptide for its respective receptor in *P. americana*.

In the current study, single replacements of amino acids at position 5 to 9 of Carmo-HrTH-II with Ala resulted in low or complete loss of potency. The large part of Carmo-HrTH-II (positions 1 to 8) consists of alternating lipophilic and hydrophilic amino acid residues, having a β-turn at position 5 to 8 (Hayes & Keeley, 1990; Zubrzycki & Gäde, 1994; Cusinato *et al.*, 1998) (Fig. 4.1). Therefore, a single replacement of these amino acids with Ala, which results in the removal of the side chain and/or the interruption of alternating hydrophilic pattern, might disrupt the peptide β-conformation. Interrupting the stability of the conformation may hinder the interaction of the peptide with its receptor through the backbone hydrogen bonding (Gäde & Hayes, 1995).

4.2. Effects of other neuropeptides and a neurotransmitter on heart beat rate.

4.2.1. The insect heart beat rate.

Two sets of semi-exposed bioassays were used to try and quantify cardio-stimulatory effects of the native hypertrehalosaemic hormones in *C. morosus*: intact and ligated/decapitated preparations. The conditions of both the preparations were the same and only differ on the relationship between the head and the rest of the insect body. In intact preparations, the heart was exposed by cutting open the abdomen of insect with the head attached on the body. In decapitated/ligated preparations, the exposed heart was done on the insect

decapitated or had the head on but ligated on the neck. The beating pattern of the heart was the same for regardless of whether the insect was intact, decapitated or ligated. The heart was beating in an anterograde and peristaltic manner with more pronounced contractions at the posterior region. This beating pattern is similar to that described for in the stick insect, *B. extradentatum* (Ejaz & Lange, 2008). The beating patterns of the dorsal vessels of stick insects (*C. morosus* and *B. extradentatum*) are similar to that of locusts (*L. migratoria*), grasshoppers (*Melanoplus differentialis* and *Schistocerca americana*) and cricket *(Acheta domestica*) but different from that of the cockroach (*P. americana*) (Miller, 1997). In *P. americana*, the dorsal vessel contracts simultaneously all along its length (Miller, 1997). In the present study, the heart beat rates of the intact and ligated/decapitated preparations were 44 and 38 beats/min, respectively, and were statistically different. The two sets of preparations were subjected to different types of stress which then resulted in different heart beat rates. Baumann *et al* (1990) reported the heart beat rate of *P. americana in vivo* to be higher than that of the semi-isolated heart (94 vs. 80 beats/min). Thus, different assays contributed the variations of among heart beat rates of insects

4.2.2. Cardio-stimulatory effects of the native hypertrehalosaemic hormones.

The first discovery of cardio-stimulatory effects of members of the AKH/RPCH family was more than 30 years ago (Scarborough *et al.*, 1984). However, the cardio-excitatory actions of the AKH/RPCH peptides have only been investigated in a number of insects. I only came across one study that investigated the structure-activity of the AKH peptides via the heart beat rate of *P. americana* (Baumann *et al*., 1990). In the present study, native hypretrehalosaemic hormones were only capable of increasing the heart beat rate of

ligated/decapitated *C. morosus* but not when intact. These results may be explained by the differences in assay preparations.

The results also indicate that the antagonistic action(s) performed against the hypretrehalosaemic hormone does not only prevent it from causing hypertrehalosaemic response but also from performing other functions including increasing the heart beat rate.

Carmo-HrTH-II was also reported to increase the heart beat rate of decapitated *B. extradentatum* (Malik *et al*., 2012). Baumann *et al*., (1990) also observed an increase in heart beat rate of *P. americana* caused by Carmo-HrTH-II in both in *in vivo* and semi-isolated heart assays. The increase in insect heart beat rate caused by AKH peptides was reported in other insects such as *Blaberus discoidalis* and *Tenebrio molitor* (Rosiński & Gäde, 1988; Keeley et al., 1991). Patel *et al*., (2014), however, reported that the heart frequency of *Rhodinius prolixus* was not changed by the endogenous AKH in an *in vitro* assay.

4.2.3. Effects of various AKH analogues on the heart of ligated *C. morosus*.

In the present, the naturally-occurring AKH peptides that were found capable of mobilising the carbohydrates in the haemolymph of *C .morosus* also stimulated the heart beat rate of this insect and vice versa. The mode of actions of the AKH peptides on the heart is still unknown. Hence, it is not known whether these peptides act directly or indirectly on the heart. However, the stimulation of the heart by the AKH peptides is believed to be the mechanism for assisting the faster distribution of fuel metabolites (Gäde & Marco, 2013).

The application of the N- or C- terminal modified analogues on the heart preparations resulted in 58% increase compared to that of Carmo-HrTH-II. The analogue that had a pGlu replaced with N-acetyl-Ala did not increase the heart beat rate. These results indicate that the native peptide may lose some of its binding affinity when the pGlu is removed, a

response that becomes more pronounced when pGlu is replaced with an acyclic residue**.** In *P. americana,* the affinity is completely lost, with no stimulation of the heart beat rate when the pGlu or amide is removed in both *in vivo* and semi-isolated heart assays (Baumann *et al.*, 1990)**.**

Single replacements of amino acids at all positions of Carmo-HrTH-II with Ala resulted in the peptide losing its efficacy completely. These data indicate that the side chains of all the amino acids of Carmo-HrTH-II are crucial for eliciting cardio-stimulatory action in *C. morosus*.

4.2.4. The effects of other neuropeptides on the heart beat rate

The present study tested the neuropeptides commonly known to have cardio-stimulatory effects in insects (reviewed by Chowański *et al.*, 2016) in order to help with the quantification of the action of native hypertrehalosaemic hormones on the heart. Previous immunohistochemistry studies on a number of insects reported the association of dense CCAP- and proctolin-like immunoactivity with the dorsal vessel, alary muscles and the ganglion as well as presence of blebs and varicosities, evidence which suggests that CCAP and proctolin are important neuropeptides for the regulation of circulation (Lehman *et al.*, 1993; Dulcis *et al.*, 2005; Ejaz & Lange, 2008). In fact, these neuropeptides have shown to impact myotropic activities, including cardio-acceleratory effects in several insects (Brown & Starratt, 1975; Lehman *et al.*, 1993; Dulcis *et al.*, 2005; Sláma & Rosiński, 2005; Ejaz & Lange, 2008). In the stick insect *Baculum extradentatum*, CCAP and proctolin were reported to be capable of increasing heart beat rate more effectively than the AKH peptides (Ejaz & Lange, 2008; Malik *et al.*, 2012). The same was observed with *C. morosus* in the present study. The increases in heart beat rate caused by CCAP and proctolin were observed at lower concentrations than those produced by Carmo-HrTH-II (i.e., 1.5 pmol for CCAP, 0.25 pmol

for proctolin and 20 pmol for Carmo-HrTH-II). Doses of 150 pmol of CCAP and 0.5 pmol of proctolin cause the heart muscles to stop contracting. The maximum increase of the heart beat rate of *C. morosus* is estimated to be 50%, because all higher doses resulted in tetanus of the heart muscle. This shows that the heart of *C. morosus* has a lower capability of increasing its contraction rate compared to *B. extradentatum* (50% vs. 461% increase, respectively) (Ejaz & Lange, 2008).

The study by Ejaz & Lange (2008) on *B. extradentatum* suggests that the cardio-acceleratory effect of CCAP is direct upon the heart and not acting through the ventral nerve cord. This direct effect explains why CCAP increased the heart beat rate of *C. morosus* regardless of whether the insect is ligated or not. In the pupae of the moth *Maduca sexta*, however, neither the injection of CCAP nor proctolin had direct cardio-stimulatory effects but instead caused delayed effects of prolonged unidirectional and faster pulsations, which occurred several hours after the injection (Sláma & Rosiński, 2005). In the fruit fly *D. melanogaster*, proctolin was found to decrease the heart beat rate for all the developmental stages *in vivo* (Zornik *et al.*, 1999).

4.2.5. Cardio-inhibitory effects of the neurotransmitter octopamine on the heart rate

Octopamine was tested in the present study with the aim of determining whether the heart of the stick insect can be inhibited. However, previous reports on the effects of octopamine on insect hearts varied. For example, in *Rhodnius prolixus*, octopamine showed *in vivo* cardio-inhibitory effects (Chiang *et al.*, 1992) while in the pupa of *D. melanogaster*, octopamine had *in vivo* cardio-stimulatory effect (Johnson *et al.*, 1997). The work of Johnson *et al.* (1997) is, however, not in agreement with that of Zornik *et al.*, (1999) who studied the

same insect. The latter study reported that octopamine decreases the heart beat rate of the pupa but stimulates that of the adult (*in vivo*).

In the present study, the application of a high concentration of octopamine (10^{-2} M) on the semi-exposed heart of *C. morosus* stimulated the heart beat rate by about 80% immediately after application for less than 2 minutes then the rate decreased to 60%. The heart then remained beating slow until octopamine was washed off 10 min after application. Lower concentrations of octopamine (10^{-3} M to 10^{-4} M) did not show the same stimulatory effects and the heart beat rate decreased right after. Chiang *et al.*, (1992) suggested that a nervous system is required for octopamine to elicit inhibitory responses on the heart. These authors suggested this requirement because octopamine showed no response on the semi-isolated heart of *R. prolixus* with abdominal nerves removed. In the current study, octopamine was applied on the heart of *C. morosus* where only the internal viscera of the abdomen were removed, but the nerves were still present.

Zornik *et al.*, (1999) claim that the disagreement between their work and that of Johnson *et al.*, (1997) was due to the differences in their *in vivo* heart bioassays. One of the differences was that the former authors injected the octopamine at the anterior side of the insect and monitored heart movement continuously while Johnson *et al.*, (1997) injected directly onto the posterior part of the heart and waited 2 min prior to recording. The present study, which observed the heart continuously after application, revealed that the effect of octopamine changed with time for some certain concentrations. With these variations among the effects of tested substances and bioassays, it is clear that more investigations (whether *in vivo* or *in vitro*) are required to fully understand the complex regulations of the insect dorsal vessel.

5. Chapter five: Concluding remarks

The present study assessed the importance of different structural features of the native HrTHs of *Carausius morosus* in interacting with the respective receptor. It supports the importance of the conserved traits of the AKH/RPCH family (i.e. blocked termini and the aromatic amino acids at position 4 and 8) in peptide-receptor binding. The study also showed that the HrTH receptor(s) for *C. morosus* are sensitive and do not tolerate many changes done on the native ligands. The study also revealed that only two AKH peptides, Phyle-CC and Rommi-CC isolated from the Orthopteran insects can function in the Phasmatodean insect. This information will contribute to the on-going effort of designing insecticides capable of hindering biological process within the specific pests.

Further studies still need to be done to investigate the hypertrehalosaemic actions of AKH/RPCH peptides in *C. morosus*. These stick insects tend to respond differently to the AKH/RPCH peptides as compared to other insects. The responses of the stick insects only match those of others insects when it is ligated at the neck. It is of great importance to investigate the possible causes of this unusual response in order to understand better the physiology of stick insects to be able to compare it to that of other insect species.

6. References

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